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13. ABSTRACT *(Maximum 200 Words)*

Physiological stress conditions associated with solid tumor play a role in chemotherapeutic resistance. Treatment with hypoxia or chemical stress agents causes EMT6 mouse mammary tumor cells to develop resistance to etoposide and teniposide, prototypic topoisomerase II inhibitors. We have shown that NFkB activation plays an important role in stress induced resistance and have used gene expression technology to identify potential genes responsible for the resistant phenotype. One pathway identified involves TGF-beta, PDGFRalpha and MEK1/2 which we have shown to be directly linked to NFkB activation in EMT6 mouse mammary tumor cells. Further additional potential genes involved in the reversal of resistance have been identified. Our data suggest that interrupting the NFkB pathway may be a useful strategy to improve the efficacy of topoisomerase II inhibitors.

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Introduction

The success of treatment for breast cancer is often limited by the development of drug resistance. We have described a unique type of resistance induced by the physiological factors at play within solid tumors. Solid tumors, due to inadequate vascularization contain microenvironmental regions that are hypoxic and thereby subject the neoplastic cells in these regions to cellular stress. The cellular response to this stress then determines cellular susceptibility to therapy. EMT6 mouse mammary tumor cells treated with hypoxia (paper in appendix) or the chemical stress agent brefedin A (BFA) induce resistance to agents that inhibit topoisomerase II (Lin et al, 1998, Brandes et al, 2001). Furthermore, data obtained through the present grant show that this chemically- and physiologic-induced resistance is mediated in large part by the nuclear transcription factor NF-κB. The overall scope of the work is to determine if NF-κB is the mediator of physiologic induced resistance and whether agents which alter NF-κB activation can alter the effectiveness of topoisomerase II type drugs.

Body

Tasks 1- 3 from **Year 1** were to insert the NF-κB p65 plasmid and then isolate, verify expression and determine drug resistance in a high and low expressor cell line. We have inserted both the p65 and p50 subunits into the ponasterone inducible vector system used previously to inhibit NfκB acivity in cells (for discussion of IκBαM results in the inducible vector system please see Brandes et al, Molecular Pharm. 2001, copy found in the appendix). Our previous data using this system has demonstrated that ponasterone A did not activate NF-κB in EMT6 cells, did not interfere with stress induced NF-κB activation, and was not toxic to the cells. Both vector p65 and p50 containing cells and empty vector cells were made for these experiments. Briefly, last year, we verified that ponasterone induced expression of p65 and p50 using western blot analysis for protein; showed that the induction of p65 and p50 in the cells led to enhanced NF-κB activation as measured by luciferase assays, and showed that p65 or p50 expressing cells were resistant to etoposide and doxorubicin, topoisomerase II inhibitors. When either p65 or p50 subunits were expressed, stress-induced activation of NF-κB was comparable to the unstressed, basal level of NF-κB activation and drug resistance was lower than in VCT (empty vector) controls. This abrogation of stress-induced activation was reflected in the lack of stress induced drug resistance during p65 or p50 expression. This type of result was surprising but has been seen in other stress pathways. For example, in the UPR pathway, mediated by unfolded protein, GRP78 (BiP) induction by stress is abrogated if in fact GRP78 is overexpressed (Dorner et al, 1987).

Additional investigation is required to determine the mechanism behind this finding. It may be that NF-κB activation by overexpressing either p65 or p50 may have simply

induced sufficient increases in IκB content in these cells such that stress could not induce NF-κB activation as efficiently. Indeed, our preliminary expression array data suggests that in fact IkB is induced during NF-κB activation as expected. We have not performed any array expression studies after p65 or p50 overexpression.

Task 4 and 5 from Year I concerned comparative experiments using an IκBαM which was not phosphorylatable and therefore could prevent the activation of NF-κB. The completed results from these studies are delineated in our paper which was published s in **Molecular Pharmacology**. A copy of the paper is in the Appendix. In brief, we showed that IκBaM expressing cells had similar sensitivities to etoposide as vector only expressing cells in the absence of stress; that stress would induced similar levels of drug resistance in both VCT and IκBaM cells (non-induced); that in IκBaM (induced)the cellular sensitivity to etoposide was similar to that of non-stressed cells and that biochemical measurements of NF-κB activation correlated with the cellular toxicity data (expression of IκBaM prevented stress-induced activation of NF-κB). This was true whether the stress used in the experiments was BFA (brefeldin A), HYP (hypoxia) or OA (okadaic acid). We conclude from the data from Task 1-5 that stress induced activation of NF-κB is the likely mechanism by which stress renders tumor cells resistant to drug induced toxicity.

In year 2, Tasks 1 and 2 were to compare drug resistance in transfected cell lines under conditions in which varying levels of expression were obtained. The original design was to isolate and use cloned with various levels of expression. To accomplish this with the inducible lines, we first attempted to determine if altering the concentration of inducing agent would alter the degree of resistance or the amount of resistance reversal that one would obtain. As seen below, our preliminary results with the IκBaM expressing cell line suggests that this is the case.

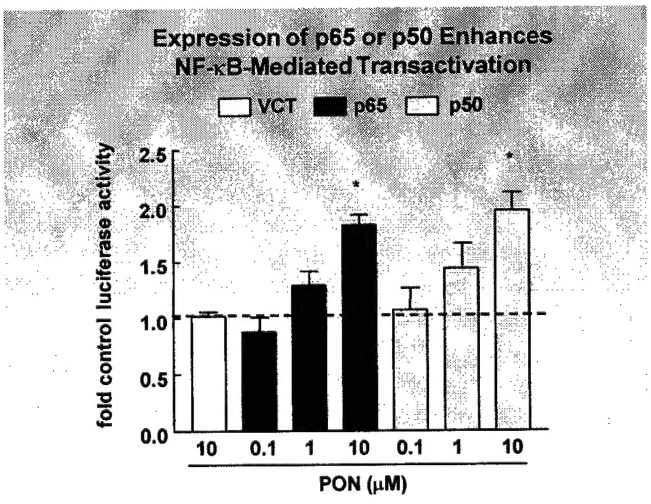
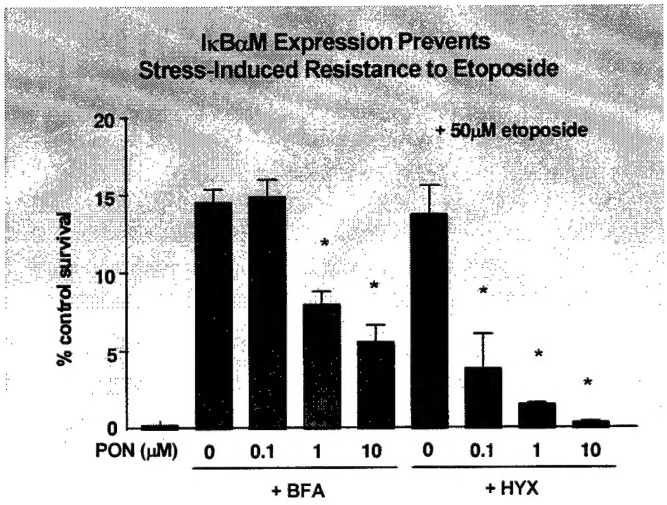


Figure A. Amount of Resistance Reversal is Dependant upon the Concentration of the Inducing Agent. IkBM cells were induced for 24 h with varying concentrations of PON and then treated with either BFA or hypoxia. Eight hours after stress treatment, cells were then treated for 1 h with

In addition,

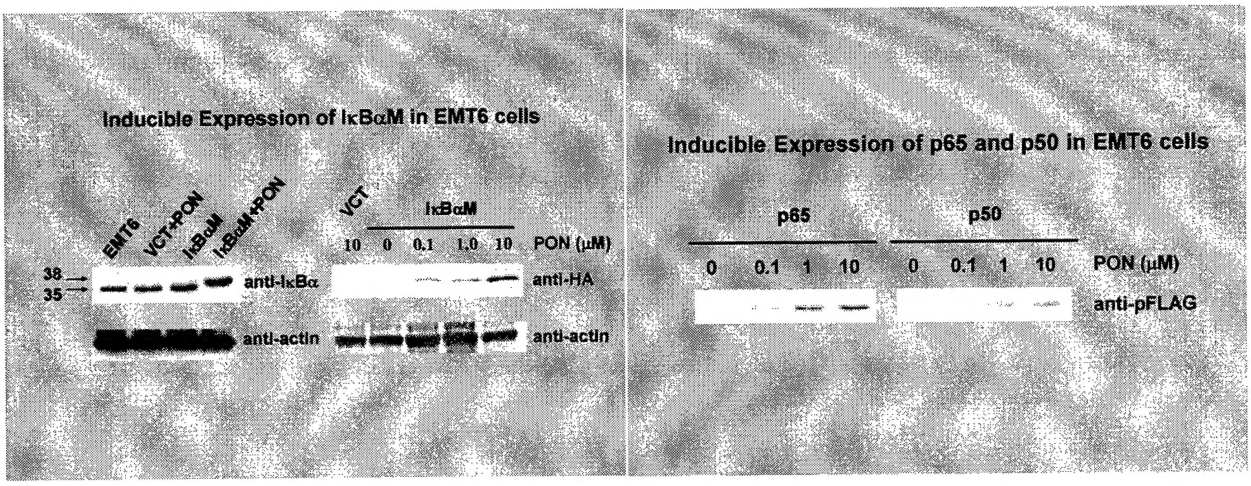
we have begun similar studies with p65 and p50 expressing cells lines as shown below in Figure B. The data also suggest that the level of resistance is dependent upon the concentration of ponasterone used for induction of the plasmid.

Figure B. Expression of p65 or p50 Induces Resistance to Etoposide. Cells were pretreated with varying concentrations of the plasmid inducing agent, Ponasterone (PON) for 24 h. The cells were then exposed to 50 μM etoposide for 1 h and cytotoxicity was assessed by colony formation. Cellular sensitivity to etoposide was dependent upon the amount of inducing agent used.



In addition, to show that induced activity is dependent upon expression, both western blot experiments and functional assays of NF-κB activity using the luciferase system were performed and showed that p65, p50 and IkBM were induced in a dose dependant manner.

Figure C: Expression of IkBMa or p65 or p50 is dependent upon the concentration of ponasterone used to induce the system. IkBMa has three HA tags and p65 or p50 has a FLAG tag. Cells were induced with various concentrations of ponasterone for 24 h and then collected for Western blot analysis.

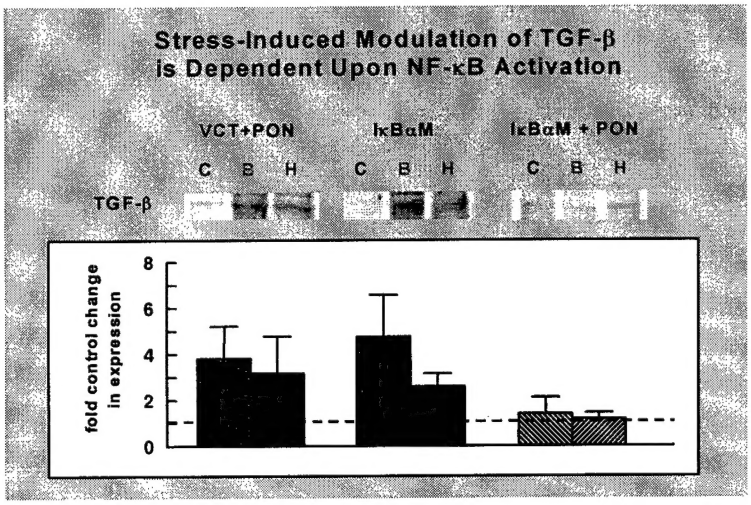


Our studies with the pharmacological inhibitor of NF-κB activation, prostaglandin A_1,(PGA_1) have been published in Oncology Research. These studies have demonstrated that stress treatment did not alter intracellular drug accumulation, topoisomerase II protein levels or inhibit topoisomerase II activity. Both hypoxia and BFA caused activation of NF-κB. Pretreatment of the cells with PGA_1 inhibited stress-induced activation of NF-κB and reversed BFA and hypoxia induced resistance. Resistance could be reversed when PGA_1 was given prior to or after the stress. These data imply that agents like PGA_1 which can abrogate the activation of NF-κB could be useful adjuncts to enhance the clinical efficacy of topoisomerase II directed chemotherapeutics. A copy of this manuscript is included in the appendix.

We have begun to determine the downstream signaling pathways that are involved in mediating resistant phenotype. The rationale behind these experiments is that understanding the downstream signals may allow us to refine the target. Because NF-κB is such a general transcription factor and plays a role in so many physiologic processes, the use of a general inhibitor may lead to a plethora of unintended effects and toxicities. Our hypothesis is that ER-stress via the ER overload pathway leads to the induction of a common subset of signals which mediate the resistance and that by comparing stresses and the reversal of stresses using expression arrays we may be able to focus in on a relatively small subset of genes. Our preliminary data which has not been fully analyzed suggests that this is the case. We have used the Affymetrix

mouse expression array system to perform initial assessments. Using matrix cross analyses of hypoxia and BFA with or without induction of IκBM induction, we can narrow the number of genes that represent the intersection of the two stresses to 40-60. In addition, initial classifications of the involved genes show that a wide variety of cell processes including cell cycle, energy metabolism, and apoptosis related genes may be involved. In addition, cursory inspection of the data show that expected gene products like IκB are increased during stress (NF-κB activation) and decreased during expression of IκBM during stress (inhibition of NF-κB activation). These data are to be presented, in a poster presentation at the ERA of Hope Meeting in Orlando FL "Exparession profiling ochemical and physiological stress treated breast tumor cells reveals a signalig pathway for drug resistance through NfkB activation" and "Expression profiling reveals a role for TGF-beta and the PDGFRa/MAPK signaling pathway in the development of stress-induced drug resistance". Copies of these posters can be found in the Appendix.

These studies have demonstrated that expression profiling is a useful methodology to assess complex changes in gene expression provided that additional methods are used to verify and corroborate the studies. For example, stress via hypoxia and brefeldin A elevated the expression of IkB as expected, VEGF, and NO synthetase. In addition we found that TGF-beta was elevated, PDGFRa and MEK 1 / 2 were decreased in expression profiles. Western blotting demonstrated that TGF-beta protein was increased, PDGFRa protein was decreased but that MEK1 and MEK2 were unaffected. However, the phosphorylated form of MEK was decreased. Additional studies with inhibitors showed that inhibiting TGF-beta, blocking the PDGFRa receptor or inhibiting the function of phosphorylated MEK all resulted in drug resistance to etoposide. To determine if these genes might be involved in NFkB dependent resistance, we assessed the status of these proteins when IkBM was induced in stressed cells. Induction of IkBM blocked the stress dependant changes in TGF-beta expression, PDGFRa, and phosphoMEK (see figure D)



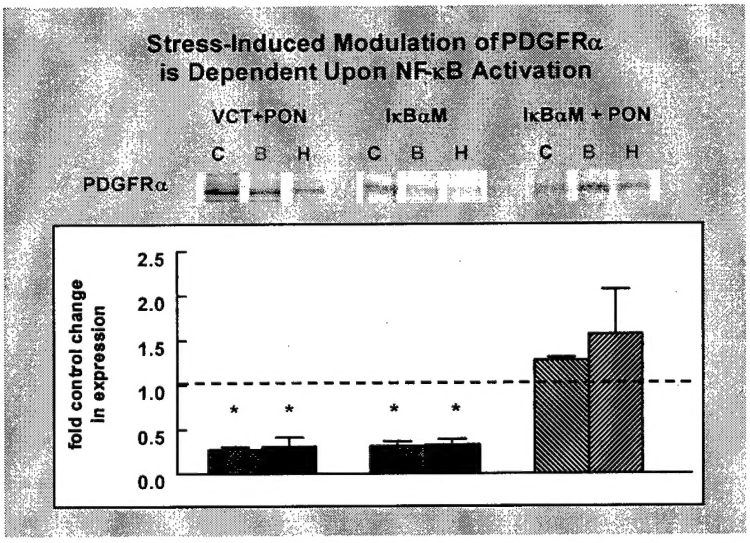
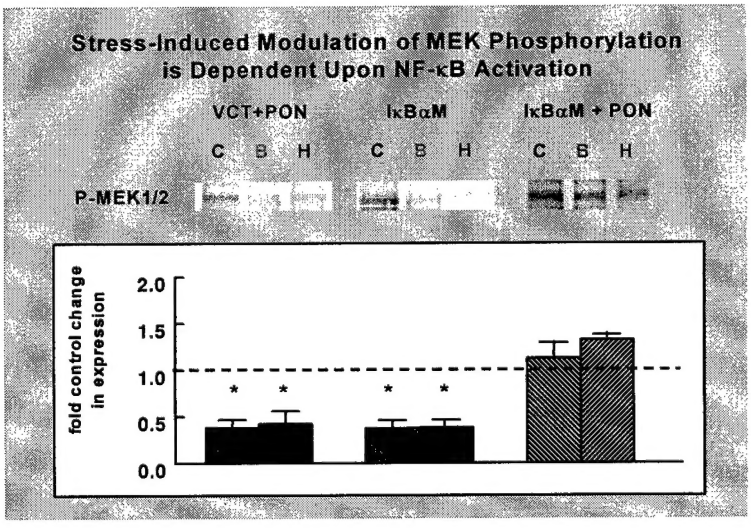


Figure D. Western blot analysis of TGF-beta, PDGFRa and phosphorylated MEK 1/ 2 in stressed EMT6 cells with or without I κBM induction with ponasterone A. Protein levels from stressed- induced empty vector cells (VCT) are shown for comparison.



Task 6 from year 1 and Tasks 3 and 4 from year 2 concern the effects of NF-κB inhibitors on *in vivo* tumors. Again this year, these studies have not begun. Although it was initially thought that the contamination of the mice from mouse hepatitis virus would be easily contained and eliminated, this has proven to be more difficult. The institution concluded that in order to maintain clean animals it would need to build a barrier facility. The facility is now complete and can be utilized. We have requested and received a one year extension of this project to allow us to complete this work.

Key Research Accomplishments

- Demonstrated that the dose dependent induction of the p65, p50 and IkBM transfected cells by ponasterone resulted in the dose dependent expression of the proteins and effects on drug resistance to etoposide.
- Showed that dose dependent induction of p65 or p50 cells by ponasterone led to increased NF-κB activity as measured by elevation of luciferase.
- Used expression arrays to begin to dissect the common downstream pathways for NF-κB mediated drug resistance.
- Demonstrated that stress induced activation of NF-κB controls TGF-beta expression in these cells and leads to a signal transduction pathway that contributes to drug resistance.
- Have identified 40-80 genes that play a possible role in mediating resistance and resistance reversal.

Reportable Outcomes

Presentations:

L.M. Brandes, S. R. Patierno, D.A. Stephan, and K.A. Kennedy, Expression analysis reveals a role for TGF-beta and the PDGFRa/MAPK signaling pathway in the development of both chemical and physiologicial induced drug resistace of breast cancer cells, Proc. Amer Assoc. Cancer Research 53: 5371, 2002

KA Kennedy, LM Brandes, and DA Stephan, Expression profiling of chemical and

physiologic stress-treated breast tumor cells reveals a signaling pathway for drug resistance through NF-κB activation, 41st Annual Meeting of the Amer. Soc. for Cell Biology, Abst. 108, 2001.

Papers:

L. M Brandes, Z.P. Lin, S. R. Patierno, and K.A. Kennedy, Reversal of physiological stress-induced resistance to topoisomerase II inhibitors using an inducible phosphorylation site-deficient mutant of IκBα, Molecular Pharm. 60: 1-9, 2001.

Y.C. Boller, L.M. Brandes, R. L. Russell, Z. P. Lin, S. R. Patierno, and K. A. Kennedy, Prostaglandin A_1 inhibits stress-induced NF-κB activation and reverses resistance to topoisomerase II inhibitors, Oncology Res. 12: 383-395, 2001.

Cell Lines:

Stable ecdysone inducible expression of p65 in the EMT6 mouse mammary tumor cell line- EMT6-p65.

Stable ecdysone inducible expression of p50 in the EMT6 mouse mammary tumor cell line- EMT6-p50.

CONCLUSIONS

Our results show that NF-κB activation by physiologic stress leads to resistance to topoisomerase II type drugs including etoposide, teniposide, and doxorubicin. Pharmacologic agents which interfere with NF-κB activation can reverse resistance when given before, during or after stress. Genetic manipulation of the IκBα subunit to block NF-κB activation render the cells completely sensitive to drug during stress. Overexpression of either the p50 or p65 subunits of the transcription factor also leads to drug resistance. Furthermore, expression analysis reveals that there is an NF-κB dependent pathway that directly influences TGF-beta expression that ultimately results in a decrease in MEK phosphorylation. These results confirmed by western blots suggest additional inhibitors that interfere with this pathway could alter the drug resistant phenotype. Indeed, pharmacologic manipulation to mimic these changes demonstrated that drug resistance was induced; the resistance that was induced was small suggesting that additional pathways downstream of NF-κB are involved in the development of the resistant phenotype. Importantly, these data show that NF-κB expression is sufficient and necessary to cause cells to become resistant to topoisomerase II inhibitors. Manipulation of tumor NF-κB activation or manipulation of the downstream signaling events arising from NF-κB activation should lead to altered

responsiveness to topoisomerase II inhibitors.

REFERENCES:

A. J. Dorner, L.C. Wasley, and R.J. Kaufman, Overexpression of GRP78 mitigates stress induction of glucose regulated proteins and blocks secretion of selective proteins in Chinese hamster ovary cells, EMBO Journal 11: 1563-1571, 1992.

Appendices:

1. L. M Brandes, Z.P. Lin, S. R. Patierno, and K.A. Kennedy, Reversal of physiological stress-induced resistance to topoisomerase II inhibitors using an inducible phosphorylation site-deficient mutant of IκBα, Molecular Pharm. 60: 1-9, 2001.

2. Y.C. Boller, L.M. Brandes, R. L. Russell, Z. P. Lin, S. R. Patierno, and K. A. Kennedy, Prostaglandin A_1 inhibits stress-induced NF-κB activation and reverses resistance to topoisomerase II inhibitors, Oncology Res. 12: 383-395, 2001.

3. LM Brandes, DA Stephan, and KA Kennedy, Expression profiling reveals a role for TGF-β and the PDGFRα/MAPK signaling pathway in the development of stress-induced drug resistance, Era of Hope Meeting, September 2002.

4. LM Brandes, DA Stephan, K. Peterson, and KA Kennedy, Expression profiling of chemical and physiological stress treated breast tumor cells reveals a signaling pathway for drug resistance through NF-κB activation, Era of Hope Meeting, September 2002.

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Reversal of Physiological Stress-Induced Resistance to Topoisomerase II Inhibitors Using an Inducible Phosphorylation Site-Deficient Mutant of IκBα

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ABSTRACT

Physiological stress conditions associated with the tumor microenvironment play a role in resistance to anticancer therapy. In this study, treatment of EMT6 mouse mammary tumor cells with hypoxia or the chemical stress agents brefeldin A (BFA) or okadaic acid (OA) causes the development of resistance to the topoisomerase II inhibitor etoposide. The mechanism of physiological stress-induced drug resistance may involve the activation of stress-responsive proteins and transcription factors. Our previous work shows that treatment with BFA or OA causes activation of the nuclear transcription factor NF-κB. Pretreatment with the proteasome inhibitor carbobenzyoxyl-leucinyl-leucinyl-leucinal inhibits stress-induced NF-κB activation and reverses BFA-induced drug resistance. To test whether NF-κB specifically mediates stress-induced drug resistance, an inducible phosphorylation site-deficient mutant of IκBα (IκBαM, S32/36A) was introduced into EMT6 cells. In this study, we show that IκBαM expression inhibits stress-induced NF-κB activation and prevents BFA-, hypoxia-, and OA-induced resistance to etoposide. These results indicate that NF-κB activation mediates both chemical and physiological drug resistance to etoposide. Furthermore, they imply that coadministration of agents that inhibit NF-κB may enhance the efficacy of topoisomerase II inhibitors in clinical cancer chemotherapy.

Solid tumors often have irregular and inadequate vascularization because of the uncontrolled cellular growth associated with tumor formation. Inadequate blood flow creates cell subpopulations within tumors that are hypoxic and/or glucose-deprived (Vaupel et al., 1989). These physiological stress conditions can result in tumor subpopulations with altered biochemical properties. Alterations such as decreased growth fraction or enhanced DNA repair can result in the development of intrinsic resistance against topoisomerase II-directed anticancer agents (Shen et al., 1987). Resistance to topoisomerase II inhibitors can also be induced by chemical stress agents that cause the inhibition of protein glycosylation, release of intracellular calcium stores, or disruption of endoplasmic reticulum (ER)-to-Golgi transport (Hughes et al., 1989; Lin et al., 1998). Taken together, these results suggest that physiological-based chemotherapeutic resistance may involve the induction of cellular stress pathways.

Under chemical or physiological stress conditions, ER function is often compromised because of the accumulation of normally folded proteins in the ER (Pahl and Baeuerle, 1997). High ER protein levels activate a cellular stress pathway known as the ER-overload response (EOR). In this pathway, ER protein overload causes the release of intracellular Ca^{2+}, formation of reactive oxygen intermediates, and activation of the nuclear transcription factor NF-κB (Pahl and Baeuerle, 1997). Evidence now shows that physiological and chemical stress agents that result in drug resistance cause the activation of the EOR pathway and the transcription factor NF-κB (Hughes et al., 1989; Pahl and Baeuerle, 1997; Lin et al., 1998). NF-κB is a heterodimeric transcription factor usually composed of the p65 and p50 DNA-binding subunits (Urban et al., 1991). Under most circumstances, NF-κB is in an inactive state, bound to an inhibitory protein, IκB, in the cytosol. Three major isoforms of IκB have been identified, of which IκBα is believed to be the predominant form (Tran et al., 1997). To activate NF-κB, IκBα is phosphorylated, ubiquitinated, and then degraded by proteasomes (Henkel et al., 1993). Point-mutation analysis has shown that IκBα is specifically phosphorylated at two resi-

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ABBREVIATIONS: ER, endoplasmic reticulum; EOR, endoplasmic reticulum-overload response; NF-κB, nuclear factor-κB; BFA, brefeldin A; OA, okadaic acid; MG-132, carbobenzyoxyl-leucinyl-leucinyl-leucinal; EMSA, electrophoretic mobility shift assay; HA, hemagglutinin; IκBαM, S32/36A mutant inhibitory nuclear factor-κB protein α; VCT, vector lacking IκBαM insert; TBST, Tris-buffered saline/Tween 20; BSA, bovine serum albumin; ANOVA, analysis of variance; UPR, unfolded protein response.

dues, serines 32 and 36, and phosphorylation site-deficient mutants are incapable of activating NF-κB (DiDonato et al., 1996). Degradation of IκB exposes a nuclear localization sequence that allows the translocation of NF-κB into the nucleus, where it then binds to κB motifs in promoter regions and directs the transcription of NF-κB–sensitive genes (Harhaj and Sun, 1999).

In addition to its role in cellular stress responses, NF-κB activation is known to protect cells from apoptosis. NF-κB activation suppresses the activation of caspase-8 through the regulation of tumor necrosis factor receptor-associated factor protein and inhibitor of apoptosis protein (Wang et al., 1998) and prevents cytochrome *c* release through activation of A1/Bfl-1, a Bcl-2 family member (Wang et al., 1999). Inhibition of NF-κB activation with expression of a mutant IκBα sensitizes tumor cells to apoptotic death by tumor necrosis factor, paclitaxel, and daunorubicin (Wang et al., 1996; Batra et al., 1999; Huang et al., 2000). Mutant IκBα expression in vivo significantly reduces growth of head and neck squamous cell carcinoma (Duffey et al., 1999) and sensitizes chemoresistant tumors to the toxic effects of camptothecin (Cusack et al., 2000). These results suggest that physiological stress-induced NF-κB activation may modulate the expression of apoptosis genes and that inhibition of NF-κB activation may prevent stress-induced drug resistance.

We have shown that EMT6 mouse mammary tumor cells treated with the chemical stress agents brefeldin A (BFA) or okadaic acid (OA) causes NF-κB activation and resistance to the topoisomerase II inhibitor teniposide (Lin et al., 1998). Treatment with BFA disrupts protein transport from the ER to the Golgi apparatus and causes activation of the EOR response (Pahl and Baeuerle, 1997). OA treatment inhibits the PP1 and PP2A phosphatases, resulting in phosphorylation of IκB and NF-κB activation (Trevenin et al., 1990). We have also shown that pretreatment with the proteasome inhibitor MG-132 inhibits NF-κB activation induced by BFA and reverses BFA-induced resistance to teniposide (Lin et al., 1998). In the present study, we show that BFA, hypoxia, and OA induce resistance to the clinically relevant topoisomerase II inhibitor etoposide. We tested whether specific inhibition of NF-κB with the phosphorylation site-deficient mutant of IκBα (IκBαM, S32/36A) prevents stress-induced NF-κB activation and reverses BFA, hypoxia-, and OA-induced resistance to etoposide. Our results show that NF-κB is a key mediator of both chemical and physiological resistance to etoposide.

Materials and Methods

Cell Culture. EMT6 mouse mammary tumor cells, provided by Dr. Sara Rockwell (Yale University, New Haven, CT), were grown in a monolayer in Waymouth's MB 752/1 medium with L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum (Sigma, St. Louis, MO), 100 units/ml streptomycin, and 25 μg/ml gentamicin sulfate (Biofluids, Rockville, MD). Cells were maintained in a humidified atmosphere of 5% CO_2/95% air at 37°C and passaged every 3 to 4 days.

Reagents and Treatments. Brefeldin A (Sigma) was dissolved in 70% ethanol to a concentration of 10 mg/ml and stored at 4°C. For electrophoretic mobility shift assay (EMSA) and luciferase assays, cells were exposed to 10 μg/ml BFA for 2 h and then incubated for 2 h in BFA-free media. For colony-forming assays, cells were exposed to BFA for 2 h and then incubated in BFA-free media for an additional 6 h. Okadaic acid (Calbiochem, La Jolla, CA) was kept at a concentration of 100 μM in dimethyl sulfoxide and stored at −20°C. Cells were treated with 60 nM OA for 8 h in all experiments. Etoposide (Sigma) at 100 μM in dimethyl sulfoxide was stored at −20°C. Cells were treated with 10 to 50 μM etoposide 1 h before clonogenicity assay. Ponasterone A (Invitrogen) was rehydrated in 70% ethanol to a concentration of 10 mM and stored at −20°C. For all experiments, cells were treated with 10 μM ponasterone A for 24 h to obtain maximal expression of IκBαM. For all assays involving hypoxia, cells were grown in 75-cm^2 glass flasks for 48 h and then exposed to continuous hypoxia as described previously (Rockwell et al., 1982) for either 2 h (EMSA and luciferase assays) or 8 h (colony-forming assays). For EMSA and luciferase assays, the length of stress treatment was chosen to correspond with the time of maximal stress-induced NF-κB activation as determined previously (Lin et al., 1998; data not shown).

Inducible IκBαM Transfection. EMT6 cells were stably transfected with a phosphorylation site-deficient mutant of IκBα (IκBαM, S32/36A, provided by Dr. Michael Karin, University of California, San Diego), which also contains three hemagglutinin (HA) tags (DiDonato et al., 1996) or a control vector lacking IκBαM (VCT) using the ecdysone-inducible expression system (Invitrogen). The IκBαM gene was first ligated into the inducible plasmid pIND to create the pIND-IκBαM plasmid. The other plasmid in the system, pVgRXR, encodes a modified ecdysone receptor and retinoid X receptor that dimerize in the presence of the inducing agent, ponasterone A, and binds response elements on the pIND plasmid. For transfection, EMT6 cells were seeded at a density of 3 to 4×10^4 cells/ml in 25-cm^2 flasks and were grown for 20 h. Cells were transfected for 1 h with 1 μg of pIND or pIND-IκBαM plasmid, 5 μg of pVgRXR plasmid, and 36 μl lipid transfection reagent (TransFast; Promega, Madison, WI) in 2.5 ml of serum-free Waymouth's media. Transfected cells were then seeded in 100 mm^2 tissue-culture dishes and treated with 400 μg/ml hygromycin B (Invitrogen) to select for incorporation of the pIND vector. Selected clones were screened by Western blot and luciferase assay for inducible IκBαM expression. Cell lines were maintained in Waymouth's media containing 400 μg/ml hygromycin and grown in Waymouth's media without hygromycin for 40 h before experimentation.

Western Blot Analysis. Cells were seeded in 100-mm^2 dishes and grown for 18 h. After treatment with ponasterone A, total cell lysates were collected by homogenizing cell pellets in 100 μl of 1× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, and 0.006% bromphenol blue). Protein lysate (20–100 μg) was mixed with 2× SDS (250 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 0.012% bromphenol blue, and 2% β-mercaptoethanol), separated on a SDS-polyacrylamide gel (4% stacking gel, pH 6.8; 10% resolving gel, pH 8.8; 30:0.8 acrylamide/bisacrylamide), and transferred to a nitrocellulose membrane by electrophoresis. After transfer, the membrane was blocked in 1× TBST (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20) with 1% bovine serum albumin (BSA) and then probed with an anti-IκBα antibody (C21; Santa Cruz Biochemicals, Santa Cruz, CA) diluted 1:1000 in 1× TBST with 1% BSA overnight at 4°C. The membrane was then washed with 1× TBST and incubated with an horseradish peroxidase-conjugated IgG anti-rabbit secondary antibody (1:10,000 dilution in 1× TBST with 1% BSA) for 1 h at room temperature. Immunoreactive bands were observed with enhanced chemiluminescent reagent (Pierce Chemical, Rockford, IL). After observation, the membrane was incubated in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 0.67% β-mercaptoethanol) at 50°C for 30 min, washed in 1× TBST for 1 h, and probed again with anti-HA and anti-actin primary antibodies (Santa Cruz Biochemicals).

Transient Transfection and Luciferase Reporter Gene Assay. We obtained a luciferase reporter plasmid, pTk-$(\kappa B)_6$-*Luc* (provided by Dr. Heike Pahl, University Hospital, Freiburg, Germany), that contains six NF-κB binding sites (κB elements) upstream of a minimal thymidine kinase promoter (Bachelerie et al., 1991). Cells

were seeded at a density of 3 to 4 × 10^4 cells/ml in 60-mm^2 dishes or 25-cm^2 flasks and grown for 20 h. Cells were transfected with 2.5 ml of serum-free Waymouth's media containing 3 μg of the luciferase reporter plasmid and 1 μg of pcDNA3.1-*lacZ* (Invitrogen) in 12 μl of reagent (TransFast; Promega). After drug treatments, cells were lysed for 15 min at room temperature in 400 μl of reporter lysis buffer (Promega) and cleared of cell debris by centrifugation. For the luciferase assay, 100 μl of luciferase assay reagent containing luciferol (Promega) was added to 20 μl of cell lysate. Light emission was measured using a Beckman scintillation counter using the single-photon monitor mode over a 1-min interval. Cells were also assayed for *lacZ* expression to correct for differences in transfection efficiency. Cell lysate (100–150 μl) was mixed with and equal amount of 2× assay buffer containing *o*-nitrophenyl-β-D-galactopyranoside (Promega) and incubated for 2 h at 37°C. Absorbance was measured at 420 nm was measured, and the relative β-galactosidase activity for each sample was used to normalize luciferase activities.

EMSA. EMSA was performed as described previously (Lin et al., 1998). Briefly, cells were seeded at a density of 3 to 4 × 10^4 cells/ml in 150-mm^2 dishes or 150-cm^2 glass flasks. After drug treatment, cells were lysed in 100 μl of lysis buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, 1 mM dithiothreitol, 0.5% Nonidet P-40, 0.5 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride) for 5 min at 4°C. Cell nuclei were separated by centrifugation at 5000 rpm for 5 min at 4°C, washed with 500 μl of washing buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, 1 mM dithiothreitol, 0.5 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride), and broken by three freeze-thaw cycles. To construct the probe, 3.5 pmol of oligonucleotide containing the NF-κB consensus sequence (Promega) was incubated with 1 μl of [γ-^{32}P]ATP (10 mCi/ml, 6000 Ci/mmol; Amersham Pharmacia Biotech, Arlington Heights, IL), 5 units of T4 polynucleotide kinase (Promega), and 10 μl of end-labeling buffer at 37°C for 1 h and then terminated with 90 μl 1× Tris/NaCl/EDTA buffer (Sigma) and passed through a G-25 spin column (Worthington Biochemicals, Freehold, NJ). Nuclear protein extract (15–20 μg) was incubated with 3 μg of poly dI · dC and 0.035 pmol of radiolabeled oligonucleotide (100,000–200,000 cpm) in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, and 4% glycerol) at room temperature for 20 min and separated on a nondenaturating 6% polyacrylamide gel (30:1 acrylamide/bisacrylamide, 0.5× Tris/borate/EDTA, and 2.5% glycerol). The resulting gel was transferred to filter paper, dried under vacuum pressure, and exposed to X-ray film.

Colony-Forming Assay. Cells were seeded in 25-cm^2 plastic flasks or 75-cm^2 glass flasks at a density of 3 to 4 × 10^4 cells/ml and grown for 18 to 40 h before treatment. VCT and IκBαM cells were treated with ponasterone A 24 h before colony assay, with stress 8 h before colony assay, and with 10 to 50 μM etoposide 1 h before colony assay. After drug treatments, cells were harvested with trypsin and serially diluted in Waymouth's medium, as described previously (Lin et al., 1998). After 7 to 10 days, colonies were stained with 0.25% crystal violet and counted. For each treatment, the percentage of control cell survival was determined by dividing the cell survival of drug-treated cells by the cell survival of appropriate nontreated or solvent-treated cells.

Statistics. For colony-forming assays, the results shown are the average percentage of control survival ± S.E.M. from three to five independent experiments, with three replicates per experiment. For luciferase assays, the results shown are the relative control luciferase activity ± S.E.M. from three to five independent experiments. Statistically significant changes in these data were determined using one-way ANOVA with multiple comparisons performed using Bonferroni's test using $p < 0.05$ (Motulsky, 1995).

Results

Treatment with BFA, Hypoxia, and OA Induce Resistance to Etoposide. Chemical and physiological stress conditions are known to activate ER stress pathways and induce resistance to topoisomerase II inhibitors (Hughes et al., 1989; Lin et al., 1998). We first determined the effect of the ER stress agents BFA, hypoxia, and OA on the clonogenic survival of etoposide-treated EMT6 cells. Cells were exposed to hypoxia for 8 h, 60 nM OA for 8 h, or 10 μg/ml BFA for 2 h, followed by recovery in BFA-free media for 6 h. Etoposide at various concentrations was added during the last hour of stress treatment before analysis by colony-forming assay. Plating efficiencies were corrected for survival changes caused by treatment with BFA (plating efficiency = 110% of control), hypoxia (plating efficiency = 80% of control), or OA (plating efficiency = 38% of control). Figure 1 shows that pretreatment with BFA, hypoxia, or OA causes greatly enhanced cell survival in the presence of etoposide compared with nonstressed cells. These data suggest that the chemical and physiological conditions known to activate the EOR pathway induce resistance to etoposide.

Inducible IκBαM Expression in EMT6 Cells. We and others have shown that chemical and physiological stress agents that cause ER stress lead to the activation of NF-κB (Lin et al., 1998; Pahl and Baeuerle, 1997). Furthermore, inhibition of NF-κB is known to enhance the toxicity of cancer chemotherapeutics (Wang et al., 1996; Batra et al., 1999; Cusack et al., 2000; Huang et al., 2000) and reverse stress-induced drug resistance (Lin et al., 1998). Therefore, we hypothesized that BFA, hypoxia, and OA cause resistance to topoisomerase II inhibitors through the activation of NF-κB. To test this hypothesis, we selected EMT6 cells transfected with either a phosphorylation site-deficient mutant of IκBα (IκBαM) or a control vector lacking IκBαM (VCT) using the ecdysone-inducible expression system. After selection in hygromycin, we screened transfectants by using Western blot analysis for expression of IκBαM after treatment with the inducing agent, ponasterone A.

The IκBαM we obtained also contains three HA tags (DiDonato et al., 1996), which cause the mutant protein to migrate more slowly than wild-type IκBα when analyzed by gel electrophoresis. Figure 2 shows that both VCT cells treated with ponasterone A and noninduced IκBαM cells

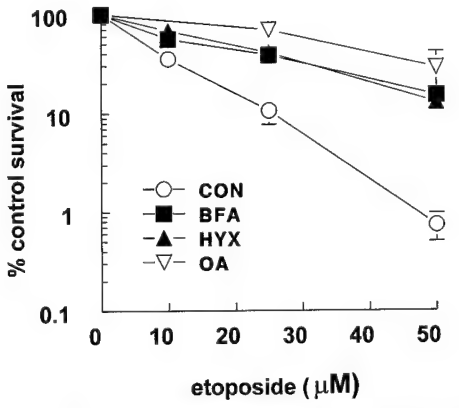

Fig. 1. Brefeldin A, hypoxia, and okadaic acid treatment cause resistance to etoposide toxicity. EMT6 cells were treated with either 10 μg/ml brefeldin A (BFA) for 2 h followed by a BFA-free recovery for 6 h, hypoxia (HYX) for 8 h, or okadaic acid (OA) for 8 h. Etoposide (10, 25, or 50 μM) was added during the last hour of stress treatment before analysis by colony-forming assay. The toxicity of etoposide in nonstressed cells (CON) is also shown. Results shown are the mean percentage of control cell survival ± S.E.M. from three to five independent experiments.

express levels of wild-type IκBα comparable with that of nontransfected EMT6 cells. However, IκBαM cells treated with ponasterone A for 24 h express the slower-migrating IκBαM only. We confirmed these findings by reprobing these blots with an anti-HA primary antibody (Fig. 2). Only ponasterone-induced IκBαM cells express protein that is immunoreactive to the anti-HA antibody. The absence of wild-type IκBα in extracts from induced IκBαM cells may be explained by the rapid association and dissociation of NF-κB/IκBα complexes (Schmid et al., 2000). Over the 24 h of ponasterone A treatment, NF-κB/IκBαM complexes become prevalent because IκBαM is not sensitive to IκB kinases and subsequent proteasome degradation (DiDonato et al., 1996). IκBα that is not bound to NF-κB, as a result of increasing competition with IκBαM, is degraded (Henkel et al., 1993) and therefore is not present at levels sufficient for detection by Western blot analysis.

IκBαM Prevents Stress-Induced Activation of NF-κB. To determine whether the expressed IκBαM was functionally active, we tested whether IκBαM expression could inhibit NF-κB activation. Cells were exposed to BFA, hypoxia, or OA stress, and nuclear extracts were prepared at times shown previously to correspond with maximal stress-induced NF-κB activation (hypoxia for 2 h, 60 nM okadaic acid for 8 h, or 10 μg/ml BFA for 2 h, followed by 2 h in BFA-free media) (Lin et al., 1998) and assayed for the presence of free NF-κB by EMSA. Our results show that BFA, OA, and hypoxia all induce NF-κB activation in both VCT cells treated with ponasterone A and noninduced IκBαM cells (Figs. 3A and 4A). In our results, two bands of specific binding are detectable, which others have suggested are the p65/p50 (upper band) and p50/p50 forms (lower band) of NF-κB (Conant et al., 1994). IκBαM cells pretreated with ponasterone A, however, had greatly reduced levels of BFA-, OA-, and hypoxia-induced NF-κB activation (Figs. 3A and 4A). To demonstrate the specificity of DNA binding, we performed competition experiments with nonlabeled NF-κB or AP-1 oligonucleotides. Figures 3B and 4B show that the addition of a 50-fold excess of NF-κB oligonucleotide effectively blocks the specific interactions of NF-κB with the radiolabeled probe. The AP-1 oligonucleotide is the same length as the NF-κB oligonucleotide, but it is otherwise not related in sequence identity. The addition of a 50-fold excess of AP-1 oligonucleotide resulted in no change in binding of NF-κB to the labeled probe (Figs. 3B and 4B). Taken together, these results show that BFA, OA, and hypoxia activate NF-κB and that IκBαM expression prevents stress-induced formation of free NF-κB in the nucleus.

To test whether IκBαM expression inhibits NF-κB function, we transiently transfected cells with an NF-κB–sensitive luciferase reporter plasmid, pTk-$(\kappa B)_6$-*Luc*. This plasmid contains a luciferase reporter gene downstream of a thymidine kinase promoter with six κB binding sites for NF-κB (Bachelerie et al., 1991). After transfection, cells were treated with ponasterone A to induce IκBαM expression and then were stress-treated (in the presence of ponasterone A) with

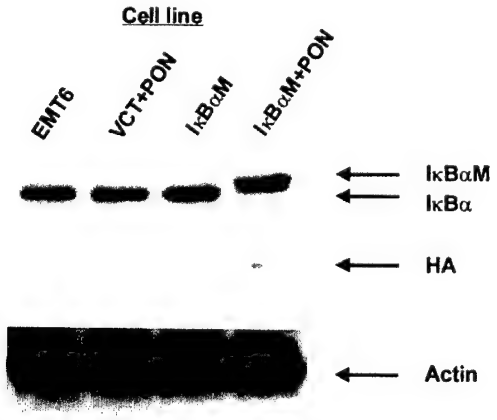

Fig. 2. Western blot analysis of inducible IκBαM expression. EMT6 cells (EMT6) were transfected with either pIND and pVgRXR (VCT) or pVgRXR and pIND-IκBαM (IκBαM). The IκBαM also contains three HA tags, which increase the molecular weight of IκBαM relative to wild-type IκBα. After treatment with 10 μM ponasterone A (PON) for 24 h, whole-cell lysates were collected and analyzed by Western blot. The resulting membranes were probed with anti-IκBα (top), anti-HA (middle), and anti-actin (bottom) primary antibodies. Shown is a representative blot from one of three independent experiments.

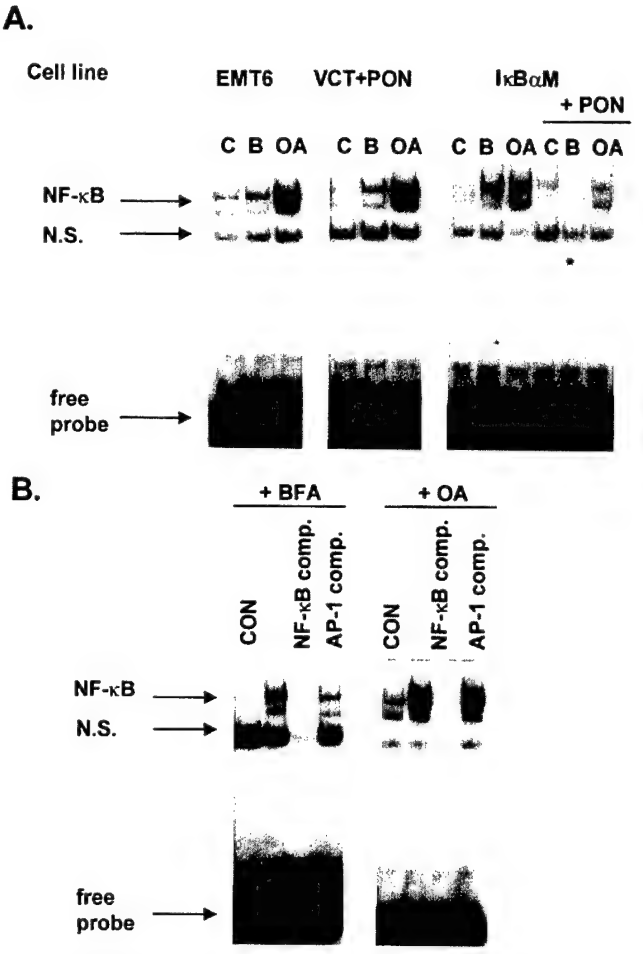

Fig. 3. A, IκBαM expression prevents chemical stress-induced activation of NF-κB. VCT and IκBαM cells were treated with 10 μM ponasterone A (PON) for a total of 24 h. VCT, IκBαM, and nontransfected EMT6 cells (EMT6) were stress-treated in the presence of PON with 10 μg/ml brefeldin A (B or BFA) for 2 h, followed by a BFA-free recovery for 2 h or 60 nM okadaic acid (OA) for 8 h (stress treatments previously shown to cause maximal NF-κB activation). After drug treatments, nuclear extracts from stress-treated and nonstressed (C) cells were harvested and analyzed by EMSA using a ^{32}P-labeled NF-κB oligonucleotide. Specific binding of NF-κB to the probe (NF-κB), nonspecific binding (N.S.), and unbound probe (free probe) bands are indicated. B, specificity of NF-κB binding by competition assay. Nuclear extracts from nonstressed (CON), BFA-, and OA-treated cells were incubated with oligonucleotide in the presence of a 50-fold excess of either unlabeled NF-κB or AP-1 oligonucleotide (NF-κB comp. and AP-1 comp., respectively).

either hypoxia for 2 h, 60 nM OA for 8 h, or 10 μg/ml BFA for 2 h, followed by a 2 h recovery in BFA-free media (time points shown previously to correspond with maximal stress-induced NF-κB activation). Cells were lysed, collected, and analyzed for luciferase expression by determining the light emission per sample in the presence of luciferol substrate. The relative luciferase activity obtained is indicative of the relative amount of functional NF-κB for a given drug treatment. Figure 5 shows that treatment of EMT6 cells with BFA, hypoxia, or OA results in a marked increase in luciferase activity compared with activity observed in nonstressed cells (Fig. 5, A and B). VCT cells treated with ponasterone A and noninduced IκBαM cells had similar increases in luciferase activity with stress treatment (Fig. 5, A and B). In contrast, IκBαM cells treated with ponasterone A had significantly less BFA-, hypoxia-, and OA-induced luciferase activity (Fig. 5, A and B). These results suggest that IκBαM expression blocks the formation of stress-induced free nuclear NF-κB and prevents enhanced NF-κB *trans*-activation caused by stress treatment.

IκBαM Expression Does Not Alter Etoposide Cytotoxicity in the Absence of Stress. Recent studies have reported that inhibition of NF-κB activation enhances the toxicity of anticancer agents (Wang et al., 1996; Batra et al., 1999; Cusack et al., 2000; Huang et al., 2000). To determine the effects of IκBαM expression on etoposide cytotoxicity in the absence of stress, VCT and IκBαM cells were induced with ponasterone A for 24 h and then treated with etoposide for 1 h before analysis by colony-forming assay. Figure 6 shows that VCT cells treated with ponasterone A, noninduced IκBαM cells, and IκBαM cells treated with ponasterone A did not have significant changes in cell survival in the

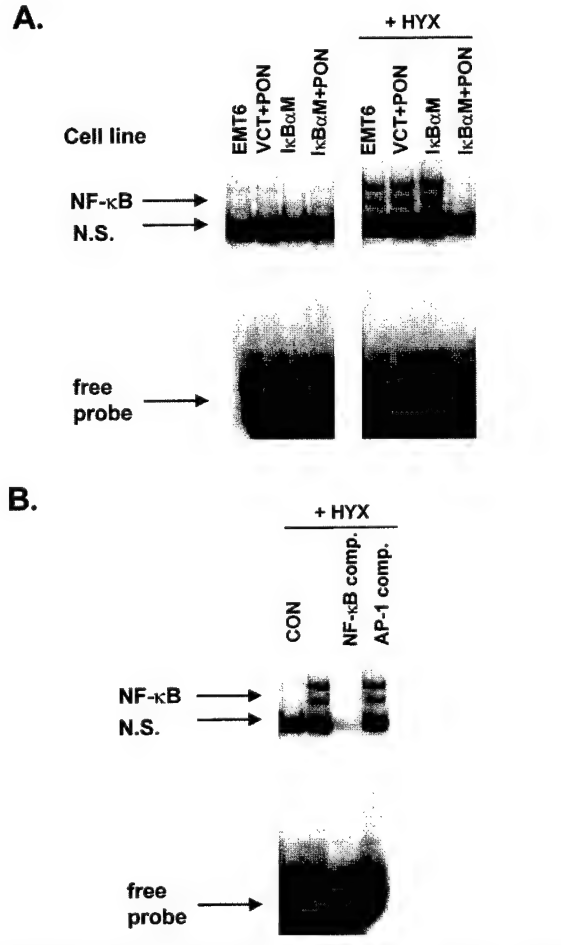

Fig. 4. A, IκBαM expression prevents hypoxia-induced activation of NF-κB. VCT and IκBαM cells were treated with 10 μM ponasterone A (PON) for 24 h. VCT, IκBαM, and nontransfected EMT6 cells (EMT6) were then stress-treated with hypoxia (HYX) for 2 h (a stress treatment previously shown to cause maximal NF-κB activation). After drug treatments, nuclear extracts were harvested and analyzed by EMSA using a ^{32}P-labeled NF-κB oligonucleotide. Specific binding of NF-κB to the probe (NF-κB), nonspecific binding (N.S.), and unbound probe (free probe) bands are indicated. B, specificity of NF-κB binding by competition assay. Nuclear extracts from nonstressed (CON) and hypoxia-treated cells were incubated with oligonucleotide in the presence of a 50-fold excess of either unlabeled NF-κB or AP-1 oligonucleotide (NF-κB comp. and AP-1 comp., respectively).

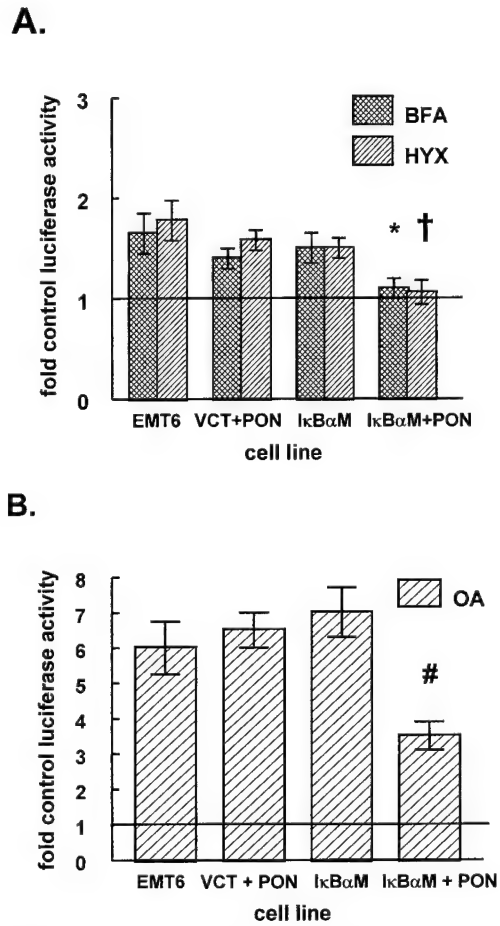

Fig. 5. IκBαM expression inhibits stress-induced NF-κB transactivation. EMT6, VCT, and IκBαM cells were transiently transfected with a NF-κB–sensitive luciferase reporter gene. After transfection, cells were treated with 10 μM ponasterone A (PON) for a total of 24 h to induce gene expression. Cells were stress-treated in the presence of M ponasterone A with either 10 μg/ml brefeldin A (BFA) for 2 h followed by a recovery in BFA-free media for 2 h, hypoxia (HYX) for 2 h (A), or 60 nM okadaic acid (OA) for 8 h (B) (time points previously shown to cause maximal NF-κB activation). Results shown are the average fold-control luciferase activities from three to five independent experiments. Bars, S.E.M. *, a statistically significant decrease in luciferase activity was observed in BFA-treated IκBαM cells induced with PON compared with noninduced BFA-treated IκBαM cells. †, a statistically significant decrease in luciferase activity was observed in hypoxia-treated IκBαM cells induced with PON compared with noninduced HYX-treated IκBαM cells. #, a statistically significant decrease in luciferase activity was observed in OA-treated IκBαM cells induced with PON compared with noninduced OA-treated IκBαM cells ($p < 0.05$, ANOVA).

presence of etoposide compared with nontransfected EMT6 cells.

IκBαM Expression Prevents BFA-, Hypoxia-, and OA-Induced Resistance to Etoposide. Our preliminary data with the proteosome inhibitor MG-132 suggested that inhibition of NF-κB activation could reverse stress-induced resistance (Lin et al., 1998). To determine whether NF-κB activation mediates stress-induced drug resistance, we assessed whether IκBαM expression could prevent BFA-, hypoxia-, and OA-induced resistance to etoposide. IκBαM cells were treated first with ponasterone A for 18 h and then with a stress treatment of hypoxia for 8 h, 60 nM OA for 8 h, or 10 μg/ml BFA for 2 h, followed by a recovery for 6 h in BFA-free media (in the continued presence of ponasterone A). During the last hour of stress, cells were treated with etoposide before analysis by colony-forming assay. Figure 7 shows that noninduced IκBαM cells treated with BFA (Fig. 7A), hypoxia (Fig. 7B), or OA (Fig. 7C) are resistant to the cytotoxic effects of etoposide. Etoposide cytotoxicity in induced VCT cells treated with stress was not statistically different from that observed in noninduced IκBαM cells treated with stress (data not shown). However, ponasterone-induced IκBαM cells treated with BFA, hypoxia, or OA (Fig. 7, ▨) were significantly more sensitive to the cytotoxic effects of etoposide compared with noninduced IκBαM cells (Fig. 7, ■). IκBαM expression partially but significantly reversed BFA-induced resistance to etoposide, whereas the reversal of hypoxia- and OA-induced drug resistance was almost complete. At nearly all doses of etoposide, the cell survival of induced IκBαM cells treated with hypoxia or OA was not significantly different from the cell survival of nonstressed IκBαM cells treated with etoposide alone. These data indicate that specific inhibition of NF-κB attenuates both chemical- and physiological-induced resistance to etoposide.

Discussion

Most solid tumors are resistant to chemotherapy. This drug resistance has been attributed, in part, to the unique physiology of solid tumors. Oxygen deficiency (hypoxia), glucose deprivation, and acidosis are widespread conditions in

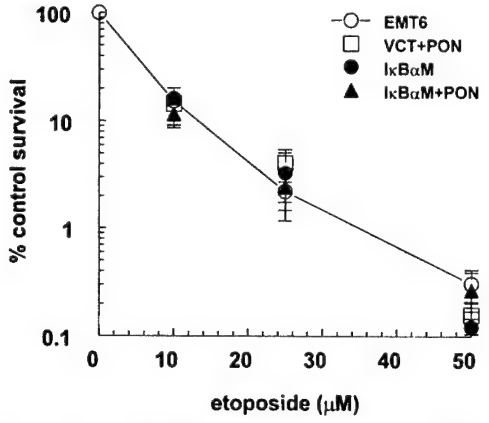

Fig. 6. IκBαM expression and stable transfection do not alter etoposide cytotoxicity in the absence of stress. VCT, IκBαM, and nontransfected EMT6 cells were treated with 10 μM ponasterone A (PON) for a total of 24 h. Etoposide (10, 25, or 50 μM) was added during the final hour of PON treatment before analysis by colony-forming assay. Results shown are the percentage of control cell survival averages of triplicate plates from at least three independent experiments. bars, S.E.M.

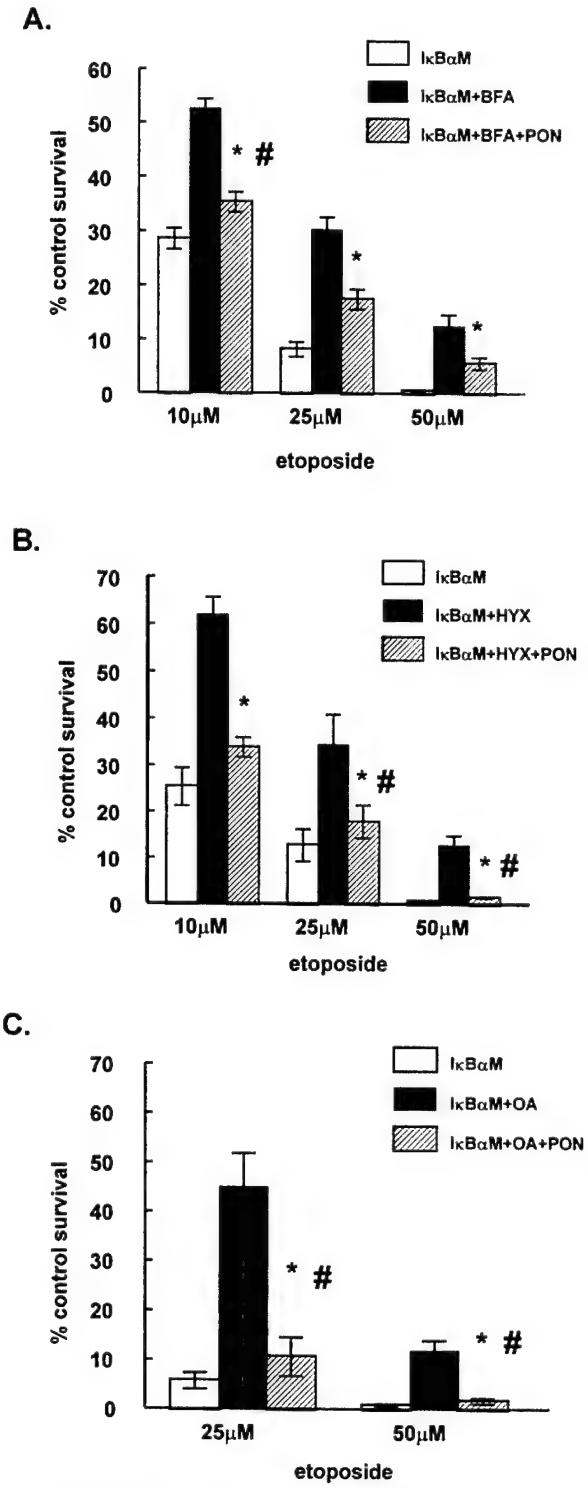

Fig. 7. IκBαM expression prevents BFA-, HYX- and OA-induced resistance to etoposide. IκBαM cells were treated with 10 μM ponasterone A (PON) for a total of 24 h. Cells were stress-treated in the presence of PON with 10 μg/ml brefeldin A (BFA) for 2 h, followed by a recovery in BFA-free media for 6 h (A), hypoxia for 8 h (B), or 60 nM okadaic acid (OA) for 8 h (C). Etoposide (10, 25, or 50 μM) was added during the final hour of PON and stress treatment before analysis by clonogenicity assay. Results shown are the percentage of control cell survival averages of triplicate plates from at least three independent experiments. Bars, S.E.M. *, a statistically significant decrease in survival of induced IκBαM cells treated with stress was observed compared with noninduced IκBαM treated with stress. #, there was no statistically significant change in survival of induced IκBαM cells treated with stress compared with nonstressed IκBαM cells ($p < 0.05$, ANOVA).

solid tumors. Hypoxia has been shown repeatedly to limit the responsiveness of tumor cells to ionizing radiation (Bush et al., 1978) and chemotherapeutic agents (Sakata et al., 1991). Mechanisms by which hypoxic cells develop resistance to radiation and chemotherapy may involve low oxygen tension and poor drug penetration into solid tumors (Durand, 1989). Hypoxia treatment is also known to cause gene amplification, cell-cycle arrest, and altered cell-cycle distribution (Stoler et al., 1992; Amellem and Pettersen, 1997). Alterations in gene expression and cell-cycle progression mediated by hypoxia-activated proteins, such as the hypoxia-inducible factor, HIF-1α (Carmeliet et al., 1998), may be involved in the resistance of tumor cells to cancer chemotherapeutic drugs.

Another type of resistance that develops in cells exposed to hypoxia may be associated with the induction of specific stress-responsive proteins and transcription factors. At the cellular level, the ER responds to stress by three distinct signaling mechanisms. One pathway, the unfolded protein response (UPR), is activated by the presence of abnormally folded proteins in the ER and results in production of the glucose-regulated protein GRP78 (Pahl, 1999). The EOR results in activation of the nuclear transcription factor NF-κB by the accumulation of normally folded proteins in the ER (Pahl, 1999). The third, the sterol regulatory cascade, is induced by the depletion of cholesterol (Pahl, 1999).

Evidence now suggests that the activation of ER stress pathways may explain the intrinsic insensitivity of solid tumors to chemotherapy. Stress conditions associated with solid tumors, such as hypoxia, induce the expression of glucose-regulated proteins (Wilson et al., 1989), heat-shock proteins (Patel et al., 1995), stress-activated protein kinases (Conrad et al., 2000), and NF-κB (Koong et al., 1998) and resistance to anticancer agents (Wilson et al., 1989). Analysis of human breast tumors has determined that GRP78 levels are elevated in malignant but not in nonmalignant lesions (Fernandez et al., 2000). The activation of ER stress responses has been further correlated with the development of resistance to anticancer agents that inhibit topoisomerase II. Treatment with the glucose-regulated stresses 2-deoxyglucose, glucosamine, calcium ionophore, or tunicamycin results in activation of NF-κB (Pahl and Baeuerle, 1997; Pahl, 1999) and the development of resistance to teniposide, etoposide, and doxorubicin (Adriamycin) (Hughes et al., 1989; Lin et al., 1998). In the present study, we show that EMT6 cells treated with other chemical stress agents, BFA or OA, or the physiological stress agent hypoxia, result in similar levels of resistance to etoposide. This finding implies that the mechanism of stress-induced resistance to etoposide may be through the activation of ER stress responses.

Our previous work suggests that the EOR pathway mediates stress-induced resistance to etoposide. We have shown that BFA treatment induces both the UPR and EOR stress pathways and causes the development of resistance to the topoisomerase II inhibitor teniposide (Lin et al., 1998). This study showed that selective activation of the EOR pathway with OA also results in the development of resistance to teniposide to an extent similar to that observed with BFA treatment (Lin et al., 1998). Selective activation of the UPR pathway with the glucosidase inhibitor castanospermine resulted in no change in sensitivity to teniposide even though it markedly increased GRP78 levels (Lin et al., 1998). Furthermore, inhibition of NF-κB activation with MG-132 or prostaglandin A_1 is sufficient to reverse BFA-induced resistance to teniposide (Lin et al., 1998; Y. C. Boller, et al., manuscript in preparation). Taken together, these data suggested the hypothesis that activation of the EOR pathway through the release of NF-κB is the mechanism by which EMT6 cells develop resistance to etoposide.

To study the role of the EOR pathway in stress-induced drug resistance, a phosphorylation site-deficient mutant of IκBα (IκBαM) was used to selectively inhibit NF-κB activation. Inducible expression of IκBαM resulted in virtually no detectable wild-type IκBα. When introduced into cells, IκBαM probably becomes the major NF-κB/IκB complex because of the high on-off rate of NF-κB/IκBα binding kinetics (Schmid et al., 2000). Over time, NF-κB/IκBαM complexes become predominant over NF-κB/IκBα complexes because IκBαM cannot be phosphorylated by IκB kinases and degraded (DiDonato et al., 1996). Free IκBα that has dissociated from NF-κB is degraded by proteasomes and thus does not appear in protein collections from IκBαM cells treated with ponasterone A for 24 h (Henkel et al., 1996).

In this study, we show that expression of IκBαM suppresses stress-induced NF-κB activation. Previous time-course studies in our laboratory have shown that maximal stress-induced NF-κB activation occurs 2 h after hypoxia treatment, 4 h after BFA treatment, and 8 h after OA treatment (Lin et al., 1998; data not shown). These time points were used in the present study to ascertain whether IκBαM expression could inhibit the maximal NF-κB response to stress. In both EMSA and luciferase reporter gene assays, VCT cells treated with ponasterone A and noninduced IκBαM cells display enhanced NF-κB activation with stress. Ponasterone A treatment alone does not activate NF-κB or interfere with stress-induced NF-κB activation in EMT6 cells (data not shown). Despite the differences in NF-κB activation kinetics, IκBαM cells treated with ponasterone A were virtually insensitive to stress-induced NF-κB activation. The expression of IκBαM also greatly inhibited NF-κB activation induced by OA, a relatively stronger activator of NF-κB (Lin et al., 1998). These data show that the inducible IκBαM was useful for testing the effects of selective inhibition of NF-κB on drug resistance.

The effects of IκBαM expression on etoposide toxicity were determined. Others have observed that IκBαM expression enhances the toxicity of anticancer agents such as camptothecin, paclitaxel, daunorubicin, and tumor necrosis factor (Wang et al., 1996; Batra et al., 1999; Cusack et al., 2000; Huang et al., 2000). However, we observed no significant change in cell survival of induced IκBαM cells treated with etoposide compared with noninduced IκBαM cells. These data suggest that NF-κB activation does not influence the cytotoxicity of topoisomerase II inhibitors in the absence of stress in our murine cell line.

We have demonstrated that inducible IκBαM expression prevents drug resistance caused by BFA, hypoxia, and OA. Noninduced IκBαM cells exhibit levels of BFA-, hypoxia-, and OA-induced resistance to etoposide similar to those levels observed in wild-type EMT6 cells. Induction of IκBαM, in contrast, results in significantly greater drug toxicity in the presence of stress. The abrogation of drug resistance was essentially complete, because hypoxia- or OA-treated IκBαM cells induced with ponasterone A had etoposide toxicity levels

that were not significantly different from those of non-stressed cells.

Taken together, our data clearly show that NF-κB activation plays a critical role in both chemical and physiological resistance to etoposide. Although statistically significant, the reversal of stress-induced drug resistance with IκBαM expression was not complete with the stress agent BFA. However, reversal of hypoxia- or OA-induced resistance was complete. Although there is evidence that IκB kinases, which play a primary role in IκB phosphorylation, may activate additional signaling pathways (Hu et al., 2001), evidence for the direct effects of IκBα on other signaling pathways is not available. Other pathways independent of NF-κB activation may contribute to stress-induced drug resistance, but the data presented here show that NF-κB activation plays a major role in stress-induced drug resistance. Our data further imply that relatively small changes in NF-κB activation can have dramatic effects on cell viability, suggesting that inhibition of NF-κB activation may result in the modulation of pleiotropic responses with biological and therapeutic significance. The concentrations of etoposide used in our studies are within the range of plasma concentrations of etoposide that are obtained clinically (Chen and Uckun, 2000). Therefore, it is likely that stress-induced resistance to topoisomerase II inhibitors play a role in the intrinsic resistance of solid tumors to topoisomerase II-directed agents. These findings also suggest that coadministration of agents that inhibit the activation of NF-κB would enhance the efficacy of topoisomerase II inhibitors in the treatment of cancer. Inhibition of NF-κB activation with IκBαM is known to enhance the toxicity of many anticancer agents both in vivo and in vitro (Wang et al., 1996; Batra et al., 1999; Cusack et al., 2000; Huang et al., 2000). Agents that inhibit NF-κB activation, such as the proteasome inhibitors PS-341 and lactacystin, enhance chemotherapeutic efficacy in in vivo tumor assays (Teicher et al., 1999; Ogiso et al., 2000). These studies and those reported here suggest that the interruption of signaling pathways mediating intrinsic drug resistance, such as physiological stress, represents new therapeutic targets for cancer drug therapy.

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References

Amellem O and Pettersen EO (1997) Cell inactivation and cell cycle inhibition as induced by extreme hypoxia: the possible role of cell cycle arrest as a protection against hypoxia-induced lethal damage. *Cell Prolif* **24:**127–141.

Bachelerie F, Alcami J, Avenzana-Seisdedos F and Virelizier JL (1991) HIV enhancer activity perpetuated by NF-κB induction of infection on monocytes. *Nature (Lond)* **350:**709–712.

Batra RK, Guttridge DC, Brenner DA, Dubinett SM, Baldwin AS and Boucher RC (1999) IkappaBalpha gene transfer is cytotoxic to squamous-cell lung cancer cells and sensitizes them to tumor necrosis factor-alpha-mediated cell death. *Am J Respir Cell Mol Biol* **21:**238–245.

Bush RS, Jenkin RD, Allt WE, Beale FA, Bean H, Dembo AJ and Pringle JF (1978) Definitive evidence for hypoxic cells influencing cure in cancer therapy. *Br J Cancer Suppl* **37:**302–306.

Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, et al. (1998) Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature (Lond)* **394:**485–490.

Chen CL and Uckun FM (2000) Highly sensitive liquid chromatography-electrospray mass spectrometry (LC-MS) method for the determination of etoposide levels in human serum and plasma. *J Chromatogr B Biomed Sci Appl* **744:**91–98.

Conant K, Atwood WJ, Traub R, Tornatore C and Major EO (1994) An increase in p50/p65 NF-κB binding to the HIV-1 LTR is not sufficient to increase viral expression in the primary human astrocyte. *Virology* **205:**586–590.

Conrad PW, Millhorn DE and Beitner-Johnson D (2000) Hypoxia differentially regulates the mitogen- and stress-activated protein kinases. Role of Ca^{2+}/CaM in the activation of MAPK and p38 gamma. *Adv Exp Med Biol* **475:**293–302.

Cusack JC, Liu R and Baldwin AS (2000) Inducible chemoresistance to 7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin (CPT-11) in colorectal cancer cells and a xenograft model is overcome by inhibition of nuclear factor-kappaB activation. *Cancer Res* **60:**2323–2330.

DiDonato J, Mercurio F, Rosette C, Wu-Li J, Suyang H, Ghosh S and Karin M (1996) Mapping of the inducible IkappaB phosphorylation sites that signal its ubiquitination and degradation. *Mol Cell Biol* **16:**1295–1304.

Duffey DC, Chen Z, Dong G, Ondrey FG, Wolf JS, Brown K, Siebenlist U and VanWaes C (1999) Expression of a dominant-negative mutant inhibitor-kappaBalpha of nuclear factor-kappaB in human head and neck squamous cell carcinoma inhibits survival, proinflammatory cytokine expression, and tumor growth in vivo. *Cancer Res* **59:**3468–3474.

Durand RE (1989) Distribution and activity of antineoplastic drugs in a tumor model. *J Natl Cancer Inst* **81:**146–152.

Fernandez PM, Tabbara SO, Jacobs LK, Manning FC, Tsangaris TN, Schwartz AM, Kennedy KA and Patierno SR (2000) Overexpression of the glucose-regulated stress gene GRP78 in malignant but not benign human breast lesions. *Breast Cancer Res Treat* **59:**15–26.

Harhaj EW and Sun SC (1999) Regulation of RelA subcellular localization by a putative nuclear export signal and p50. *Mol Cell Biol* **19:**7088–7095.

Henkel T, Machleidt T, Alkalay I, Kronke M, Ben-Neriah Y and Baeuerle PA (1993) Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. *Nature (Lond)* **365:**182–185.

Hu Y, Baud Y, Oga T, Kim KI, Yoshida K and Karin M (2001) IKKalpha controls formation of the epidermis independently of NF-kappaB. *Nature (Lond)* **410:**710–714.

Huang Y, Johnson KR, Norris JS and Fan W (2000) Nuclear factor-kappaB/IkappaB signaling pathway may contribute to the mediation of paclitaxel-induced apoptosis in solid tumor cells. *Cancer Res* **60:**4426–4432.

Hughes CS, Shen JW and Subjeck JR (1989) Resistance to etoposide induced by three glucose-regulated stresses in Chinese hamster ovary cells. *Cancer Res* **49:**4452–4454.

Koong AC, Chen EY, Mivechi NF, Denko NC, Stambrook P and Giacca AJ (1998) Hypoxic activation of nuclear factor-kappa B is mediated by a Ras and Raf signaling pathway and does not involve MAP kinase (ERK1 or ERK2). *Cancer Res* **54:**5273–5279.

Lin ZP, Boller YC, Amer SM, Russell RL, Pacelli KA, Patierno SR and Kennedy KA (1998) Prevention of brefeldin A-induced resistance to teniposide (VM26) by the proteosome inhibitor MG-132: involvement of NF-κB activation in drug resistance. *Cancer Res* **58:**3059–3065.

Motulsky H (1995) *Intuitive Biostatistics*, pp 258–259, Oxford University Press, New York.

Ogiso Y, Tomida A, Lei S, Omura S and Tsuruo T (2000) Proteasome inhibition circumvents solid tumor resistance to topoisomerase II-directed drugs. *Cancer Res* **60:**2429–2434.

Pahl H (1999) Signal transduction from the endoplasmic reticulum to the cell nucleus. *Physiol Rev* **79:**683–701.

Pahl HL and Baeuerle PA (1997) The ER-overload response: activation of NF-kappa B. *Trends Biochem Sci* **22:**63–67.

Patel B, Khaliq A, Jarvis-Evans J, Boulton M, Arrol S, Mackness M and McLeod D (1995) Hypoxia induces HSP 70 gene expression in human hepatoma (HEP G2) cells. *Biochem Mol Biol Int* **36:**907–912.

Rockwell S, Kennedy KA and Sartorelli AC (1982) Mitomycin-C as a prototype bioreductive alkylating agent: in vitro studies of metabolism and cytotoxicity. *Int J Radiat Oncol Biol Phys* **8:**753–755.

Sakata K, Kwok TT, Murphy BJ, Laderoute KR, Gordon GR and Sutherland RM (1991) Hypoxia-induced drug resistance: comparison to P-glycoprotein-associated drug resistance. *Br J Cancer* **64:**809–814.

Schmid JA, Birbach A, Hofer-Warbinek R, Pengg M, Burner U, Furtmuller PG, Binder BR and deMartin R (2000) Dynamics of NF-kappaB and IkappaBalpha studied with green fluorescent protein (GFP) fusion proteins. Investigation of GFP-p65 binding to DNA by fluorescence resonance energy transfer. *J Biol Chem* **275:**17035–17042.

Shen J, Hughes C, Chao C, Cai J, Bartels C, Gessner T and Subjeck J (1987) Coinduction of glucose-regulated proteins and doxorubicin resistance in Chinese hamster cells. *Proc Natl Acad Sci USA* **84:**3278–3282.

Stoler DL, Anderson GR, Russo CA, Spina AM and Beerman TA (1992) Anoxia-inducible endonuclease activity as a potential basis of the genomic instability of cancer cells. *Cancer Res* **52:**4372–4378.

Teicher BA, Ara G, Herbst R, Palombella VJ and Adams J (1999) The proteasome inhibitor PS-341 in cancer therapy. *Clin Cancer Res* **5:**2638–2645.

Tran K, Merika M and Thanos D (1997) Distinct functional properties of IkappaB alpha and IkappaB beta. *Mol Cell Biol* **17:**5386–5399.

Trevenin C, Kim S-J, Rieckmann P, Fujiki H, Norcross MA, Sporn MB, Fauci AS and Kehrl JH (1990) Induction of nuclear factor-κB and the human immunodeficiency virus long terminal repeat by okadaic acid, a specific inhibitor of phosphatase 1 and 2A. *New Biol* **2:**793–800.

Urban MB, Schreck R and Baeuerle PA (1991) NF-κB contacts DNA by a heterodimer of the p50 and p65 subunits. *EMBO (Eur Mol Biol Organ) J* **10:**1817–1825.

Vaupel P, Kallinowski F and Okunieff P (1989) Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer Res* **49:**6449–6465.

Wang CY, Guttridge DC, Mayo MW and Baldwin AS (1999) NF-kappaB induces

expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. *Mol Cell Biol* **19:**5923–5929.

Wang CY, Mayo MW and Baldwin AS (1996) TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. *Science (Wash DC)* **274:**784–787.

Wang CY, Mayo MW, Korneluk RG, Goeddel DV and Baldwin AS (1998) NF-kappaB and antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science (Wash DC)* **281:**1680–1683.

Wilson RE, Keng PC and Sutherland RM (1989) Drug resistance in Chinese hamster ovary cells during recovery from severe hypoxia [published erratum appears in *J Natl Cancer Inst* **82:**239 (1990)]. *J Natl Cancer Inst* **81:**1235–1240.

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Prostaglandin A_1 Inhibits Stress-Induced NF-κB Activation and Reverses Resistance to Topoisomerase II Inhibitors

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Stress conditions associated with solid tumors lead to the formation of heterogeneous tumor cell subpopulations and insensitivity to cancer chemotherapeutics. In this report, we show that EMT6 mouse mammary tumor cells treated with the chemical stress, brefeldin A (BFA), or the physiological stress, hypoxia, develop resistance to the topoisomerase II (topoII) inhibitors teniposide and etoposide. BFA and hypoxia treatment did not alter intracellular drug concentrations, topoII protein levels, or inhibit topoII activity. BFA and hypoxia did cause the activation of the nuclear transcription factor NF-κB. We demonstrate that pretreatment with the synthetic cyclopentenone prostaglandin A_1 (PGA_1) inhibits stress-induced NF-κB activation and reverses BFA- and hypoxia-induced resistance. The reversal of BFA-induced resistance can occur when PGA_1 is administered either before or several hours after the induction of stress. Taken together, these data support the involvement of NF-κB in stress-induced drug resistance, show that pharmacologic inhibitors of NF-κB can disrupt the biological consequences of stress, and imply that inhibitors of NF-κB may be useful agents to enhance the clinical efficacy of topoII-directed chemotherapeutics.

Key words: Nuclear factor-κB; Prostaglandin A_1; Drug resistance; Stress; Etoposide

The development of drug resistance is a major obstacle to the successful treatment of human cancers. Resistance of tumors to chemotherapeutic agents that inhibit the topoisomerase II (topoII[4]) enzyme is often an acquired phenotype that develops in response to repeated drug administration (1). The most common mechanism of acquired resistance is through expression of the 170-kDa P-glycoprotein or 190-kDa transmembrane transport protein, which prevents intracellular drug accumulation (2,3). Resistance to topoII inhibitors has also been correlated with decreased levels of topoII or decreased topoII activity (4).

A nonacquired form of resistance to topoII-directed agents also has been identified. Solid tumors often have inadequate vascularization due to the progressive expansion of malignant cells. Poor blood flow causes the formation of hypoxic, acidic and/or nutrient-deprived cell subpopulations that can have altered growth and functional properties (5,6). Stress conditions such as glucose deprivation and hypoxia are known to cause intrinsic tumor cell resistance to topoII inhibitors (7–9). We and others have demonstrated that intrinsic resistance to topoII inhibitors can also be induced by the activation of stress-responsive proteins through the inhibition of protein glycosylation, release of endoplasmic reticulum (ER) calcium stores, or disruption of ER-to-Golgi transport (10,11). These findings suggest that the pathways activated by ER stress responses may mediate physiologically induced resistance to topoII inhibitors.

Resistance to topoII inhibitors has been associated with the induction of the 78-kDa glucose-regulated stress protein (GRP78). Treatment with 2-deoxyglucose, glucosamine, tunicamycin, brefeldin A (BFA), calcium ionophores, glucose deprivation, or hypoxia has been correlated with the induction of GRP78 and the development of intrinsic resistance to topoII inhibitors (10–13). However, evidence now suggests that GRP78 induction is not sufficient to cause drug resistance. We have shown EMT6 mouse mammary tumor cells treated with the glucosidase inhibitor castanospermine exhibited elevated GRP78 levels but remained sensitive to teniposide (11). Inhibition of GRP78 with an antisense oligodeoxynucleotide resulted in no alteration in stress-induced apoptosis by the glycosylation inhibitor tunicamycin (13).

Another ER stress response pathway has been characterized that involves the nuclear transcription factor NF-κB. NF-κB is a heterodimeric transcriptional activator protein often bound to a cytosolic inhibitory IκB protein (14–16). To activate NF-κB, IκB must be phosphorylated, ubiquitinated, and then degraded by proteasomes (15,16). After degradation of IκB, NF-κB translocates into the nucleus where it binds to consensus elements and directs the transcription of NF-κB-depen-

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[4]Abbreviations used: topoII, topoisomerase II; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; BFA, brefeldin A; NF-κB, nuclear factor-κB; IκB, inhibitory NF-κB protein; EOR, ER overload response; MG-132, carbobenzyoxyl-leucinyl-leucinyl-leucinal; PGA_1, prostaglandin A_1; OA, okadaic acid; kDNA, kinetoplast DNA; TK, thymidine kinase.

dent genes (17). Our laboratory and others have shown that a variety of physiological and chemical stress conditions result in the activation of NF-κB (11,18). Accumulation of protein in the ER by stress causes the release of intracellular Ca^{2+} from the ER, the formation of reactive oxygen species, and the activation of NF-κB by the ER-overload pathway (EOR) (19).

NF-κB activation is also known to protect cells from apoptosis and the toxic effects of anticancer agents. NF-κB activation inhibits caspase 8 activation through the regulation of tumor necrosis factor receptor-associated factor (TRAF) and inhibitor of apoptosis (IAP) proteins (20) and prevents cytochrome c release through activation of the A1/Bfl-1 gene product, a Bcl-2 family member (21). Inhibitors of NF-κB activation are known to enhance the toxicity of tumor necrosis factor, paclitaxel, daunorubicin, and camptothecin on tumor cells (22–24). These findings suggest that stress-induced NF-κB activation may cause resistance to topoII inhibitors by preventing apoptosis and that inhibition of NF-κB activation may reverse stress-induced drug resistance.

We have shown that treatment with the chemical stress agent BFA causes NF-κB activation and the development of resistance to the topoII inhibitor teniposide (11). BFA induces stress through the EOR pathway by disrupting protein transport from the ER to the Golgi apparatus (25). Pretreatment with the proteasome inhibitor MG-132 (carbobenzyoxyl-leucinyl-leucinyl-leucinal) blocks BFA-induced activation of NF-κB and reverses BFA-induced resistance to teniposide (11). To extend these findings, we investigated the effects of another pharmacologic inhibitor of NF-κB activation on stress-induced drug resistance. Prostaglandin A_1 (PGA_1) is a synthetic cyclopentenone prostaglandin that has been used therapeutically to inhibit DNA and RNA viruses and prevent the growth of a variety of tumors (26,27). PGA_1 is also a potent inhibitor of NF-κB activation by preventing phosphorylation of IκB and causing upregulation of IκB (28,29).

In this study, we demonstrate treatment with the chemical stress, BFA, or the physiologic stress, hypoxia, results in NF-κB activation and resistance of EMT6 mouse mammary tumor cells to teniposide and etoposide. BFA or hypoxia treatment alone did not alter intracellular etoposide accumulation, topoII levels, or topoII activity, suggesting stress-induced resistance is not mediated by the common mechanisms of acquired drug resistance. To investigate the role of NF-κB in stress-induced resistance, we have tested if a pharmacologic inhibitor of NF-κB could reverse BFA- and hypoxia-induced resistance. Pretreatment with PGA_1 inhibits stress-induced activation of NF-κB and reverses BFA- and hypoxia-induced resistance to teniposide and etoposide. Furthermore, we demonstrate that BFA-induced resistance can be reversed with PGA_1 even when the reversal agent is given several hours after the induction of stress.

MATERIALS AND METHODS

Cell Culture

EMT6 mouse mammary tumor cells (provided by Dr. S. Rockwell, Yale University, New Haven, CT) were grown in a monolayer in Waymouth's MB 752/1 medium with L-glutamine (Invitrogen, San Diego, CA) supplemented with 15% fetal bovine serum (Sigma, St. Louis, MO), 100 μg/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml gentamicin sulfate (Biofluids, Rockville, MD). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air and passaged every 3–4 days.

Reagents and Treatments

To induce stress, cells were treated with 10 μg/ml BFA (Sigma) for 2 h followed by an incubation in BFA-free medium (luciferase assay and EMSA) or for 2 h followed by an incubation in BFA-free medium for 6 h (all other experiments). Cells were also treated with 5 μg/ml tunicamycin (Sigma) for 6 h, 10 mM 2-deoxy-D-glucose (Sigma) for 18 h, 10 mM glucosamine (Sigma) for 18 h, or 60 nM okadaic acid (OA) (Calbiochem, San Diego, CA). For hypoxia, cells were seeded in gas-impermeable glass flasks and then exposed to continuous hypoxia as described previously (30) for either 2 h (luciferase assay and EMSA), 8 h (colony-forming experiments), or 18 h (drug uptake and Western blot). To reverse drug resistance, 25 μM PGA_1 (Biomol, Plymouth Meeting, PA) was added 0.5 h prior to the induction of stress, concurrent with the addition of BFA, or 30 min, 1, 2, 4, 6, or 7 h after the induction of BFA stress. Teniposide (Bristol Myers, Syracuse, NY) and etoposide (Sigma) were added during the final hour of stress treatment prior to analysis by colony-forming assay.

Drug Uptake Measurements

Cellular uptake of radiolabeled etoposide was measured as previously described (31). During the final hour of stress treatment cells were incubated in 50 μM unlabeled etoposide, 2.5 μCi (2.78 nM) of ^{3}H-labeled etoposide (900 mCi/mmol, Moravek Biochem, Brea, CA), and 0.5 μCi of ^{14}C-labeled inulin. To ensure complete removal of extracellular ^{3}H-labeled etoposide, radiolabeled inulin levels were monitored. Unlabeled etoposide (50 μM) was added to the washes to prevent loss of intracellular etoposide stores. After washing, cells were lysed in 1 N NaOH, neutralized with 1 N HCl, plated in Ecolite scintillation cocktail (ICN, Costa Mesa, CA), and counted by scintillation spectrometry. For each experiment, cell counts were performed on triplicate tissue culture plates for each stress treatment and radiolabeled etoposide uptake was expressed as picomoles per 10^6 cells.

Whole Cell and Nuclear Extraction of TopoII

Following stress treatment, whole cell lysates were collected by suspending cell pellets in 90 μl of 1× DNase/RNase (with 10 μg/ml each of antipain, aprotonin, chymotrypsin, leupeptin, and pepstatin A and 35 μg/ml PMSF) and 10 μl 10% SDS. After incubation on ice for 60 min, 60 μl of SDS (8% SDS, 200 mM Tris-Cl, 40% glycerol, 400 mM DTT) was added and the samples were boiled for 5 min. For nuclear extraction, cells were washed in buffer A (0.15 M NaCl, 10 mM

KH_2PO_4, pH 7.5) and incubated for 20 min in buffer B (10 mM Tris, 1.5 mM $MgCl_2$, 10 mM NaCl, 4 mM DTT, and 1 mM PMSF, pH 7.5) at 4°C. Next, 1 ml of Nonidet detergent was added and the cells were gently triturated and incubated at 4°C for 15 min. Cells were lysed using dounce homogenization for 20 strokes and then centrifuged at $2500 \times g$ for 10 min. The pellet was resuspended in 2 ml of buffer C (50 mM Tris, 25 mM KCl, 2 mM $CaCl_2$, 3 mM $MgCl_2$, 0.25 M sucrose, 4 mM DTT, 1 mM PMSF, pH 7.5) and then layered over buffer D (buffer C with 0.6 M sucrose). Cell nuclei were isolated by centrifugation at $6000 \times g$ for 10 min at 4°C. The nuclear pellet was resuspended in 1 ml of buffer E (50 mM Tris, 25 mM KCl, 3 mM $MgCl_2$, 4 mM DTT, 1 mM PMSF, pH 7.5) and incubated for 15 min at 4°C. An equal volume of buffer F (50 mM Tris, 2 M NaCl, 4 mM DTT, 10 mM disodium EDTA, 1 mM PMSF, pH 7.5) was added and the samples were incubated for 30 min at 4°C. Finally, 10% glycerol was added and the protein extracts were collected after ultracentrifugation at $100,000 \times g$ for 60 min.

Western Blot Analysis

Nuclear extract (50–100 μg) or whole cell lysate (2 mg total protein) was mixed with 2× SDS sample buffer (250 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 0.012% bromophenol blue, 2% β-mercaptoethanol) and boiled for 5 min. Proteins were separated on a 10% SDS-polyacrylamide gel (4% stacking gel, pH 6.8, 10% resolving gel, pH 8.8, 30:0.8 acrylamide/bisacrylamide) and transferred to a nitrocellulose membrane. After blocking in 1× TBST (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) with 5% nonfat dry milk, the membrane was incubated with a 1:1000 dilution of primary polyclonal rabbit antibody against p170 topoII (provided by Dr. D. Sullivan, University of S. Florida, Tampa, FL) at 4°C. The blot was washed with 1× TBST and incubated with a secondary goat anti-rabbit HRP-conjugated antibody (Promega, Madison, WI) at a dilution of 1:10000 for 1 h at room temperature. Antibody binding was then visualized using an enhanced chemiluminescent reagent (Amersham, Arlington Heights, IL).

In Vitro Human TopoII Assay

Reaction mixtures of 2 units of purified human topoIIα enzyme, 200 ng kinetoplast DNA (kDNA), 1× assay buffer [0.05 M Tris-Cl (pH 8.0), 0.12 M KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, and 30 μg/ml bovine serum albumin] (TopoGEN, Columbus, OH), and either 25 μM PGA_1, 10 μg/ml BFA, or 100 μM teniposide were combined and incubated at 37°C for 1 h. After termination with 10 μl of stop solution (5% sarkosyl, 0.0025% bromophenol blue, and 25% glycerol), decatenation products were visualized by gel electrophoresis on an agarose gel with ethidium bromide and quantitated using an Eagle Eye transilluminator (Stratagene, La Jolla, CA). Percent decatenation was determined by dividing the relative intensity of decatenation product bands by the total lane intensity.

Electrophoretic Mobility Shift Assay (EMSA)

Following drug treatment, nuclear extracts were prepared from 1×10^7 cells as previously described (11). Briefly, cells were incubated in 100 μl of lysis buffer containing 10 mM HEPES (pH 7.9), 1 mM EDTA, 60 mM KCl, 1 mM DTT, 0.5% NP-40, 0.5 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. Cell nuclei were washed in 500 μl nuclear washing buffer (lysis buffer without NP-40 added), resuspended in 100 μl of nuclear resuspension buffer [250 mM Tris-HCl (pH 7.8), 60 mM KCl, 1 mM DTT, 0.5 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride] and broken by three freeze–thaw cycles. An oligonucleotide of the NF-κB consensus site (Promega) was labeled with 10 mCi/ml [γ-^{32}P]ATP (6000 Ci/mmol; Amersham, Arlington Heights, IL/Andotek, Irvine, CA) using T4 polynucleotide kinase (Promega) and cleared of unincorporated nucleotides by chromatography through a G-25 spin column (Worthington, Plymouth Meeting, PA). Nuclear extracts (10 μg) were incubated with 4 μg poly(dI-dC) (Sigma) and 0.035 pmol (50,000–200,000 cpm) of probe in a buffer of 10 mM Tris-HCl (pH 7.5), 50 mM sodium chloride, 0.5 mM EDTA, 1 mM magnesium chloride, 0.5 mM DTT, and 4% glycerol at room temperature for 20 min. Binding was analyzed by autoradiography after electrophoresis on a nondenaturing 6% polyacrylamide gel.

Transient Transfection and Luciferase Reporter Gene Assay

Transfection medium containing 3 μg of pTK-6κB-*Luc* plasmid (provided by Dr. Heike Paul, University Hospital Freiburg, Germany) (32), 1 μg of pcDNA3.1-*lacZ* (Invitrogen), 12 μl of transFast lipid (Promega), and 2.5 ml of serum-free Waymouth's medium was incubated with cells for 2 h. Following transfection, 2.5 ml of Waymouth's medium containing 30% serum was added. After drug treatments, cells were lysed in 400 μl Reporter Lysis Buffer (Promega) and 20 μl of whole cell lysate was combined with 100 μl Luciferase Assay Reagent containing luciferol (Promega). Light emission in the presence of substrate was measured using a scintillation counter (Beckman). Cell extracts were also assayed for *lacZ* expression by incubating 150 μl of lysate and 150 μl of 2× assay buffer containing *o*-nitrophenyl-β-D-galactopyranoside (Promega) at 37°C for 2 h. The absorbance at 420 nm was measured for each sample and the relative β-galactosidase activity was determined by dividing the activity of drug-treated cell extracts by the activity of solvent-treated cell extracts and then used to correct for variations in transfection efficiency. The fold change in luciferase activity was then calculated by dividing the corrected luciferase activity of drug-treated cells by the corrected luciferase activity of solvent-treated cells.

Colony-Forming Assay

Following drug treatment, cells were serially diluted in Waymouth's medium, seeded in triplicate 60-mm^2 tissue culture dishes, and incubated under maintenance conditions for 7 days as previously described (11). Following incubation, dishes were stained with 0.25% crystal violet and counted. For each treatment, the percent control cell survival was determined by dividing the plating efficiency for a given treatment by the plating

efficiency of the appropriate nontreated or solvent-treated control.

Statistics

Statistically significant changes in these data were determined using one-way analysis of variance (ANOVA) with multiple comparisons performed using Bonferroni's test with $P < 0.05$ (33).

RESULTS

Stress conditions associated with tumor microenvironments are known to cause resistance to cancer chemotherapeutics (8–11). To determine whether stress alters the cytotoxicity of topoII inhibitors, we exposed EMT6 cells to the chemical stress agent, BFA, or the physiological stress, hypoxia, prior to treatment with teniposide and etoposide. EMT6 cells were treated with hypoxia for 8 h or with 10 μg/ml BFA for 2 h, followed by an incubation in BFA-free medium for 6 h. Teniposide and etoposide were added during the last hour of stress treatment prior to analysis by colony-forming assay. Figure 1 shows that cells pretreated with BFA (Fig. 1A and B) or hypoxia (Fig. 1C) are resistant to the toxicity of teniposide and etoposide.

Our previous data with the proteasome inhibitor MG-132 suggest that NF-κB activation may regulate stress-induced drug resistance (11). To explore the role of NF-κB activation in stress-induced resistance, we treated cells with 25 μM PGA_1 30 min prior to the introduction of stress with BFA and hypoxia. Figure 1 shows that pretreatment with PGA_1 prevents BFA-induced resistance to teniposide and etoposide and hypoxia-induced resistance to teniposide. PGA_1 treatment alone did not alter drug toxicity in the absence of stress (Fig. 1).

A common mechanism of resistance to topoII inhibitors is through enhanced drug efflux (2,3). To test whether stress-induced drug resistance is explained by a decrease in drug concentration, we determined if BFA or hypoxia treatment alters intracellular drug accumulation of radiolabeled etoposide. To allow time for the protein-mediated effects of stress treatment on drug uptake, we treated EMT6 cells with hypoxia for 18 h. Preliminary time course studies in our laboratory have determined that the level of stress-induced resistance to teniposide with 18 h of hypoxia is not statistically different than that observed with 8 h of hypoxia (34). During the final hour of stress treatment ^3H-labeled etoposide was added. After washing, cell samples were lysed and analyzed for ^3H incorporation by scintillation spectroscopy. Our results show that treatment with BFA (10 μg/ml for 2 h, followed by a BFA-free recovery for 6 h) or hypoxia (18 h) does not result in any significant change in intracellular etoposide concentration (Fig. 2).

Normal cellular concentrations of topoII are necessary for the cytotoxicity of topoII inhibitors (4). We have investigated whether stress-induced drug resistance is explained by alterations in total cellular or nuclear levels of the topoII enzyme. EMT6 cells were exposed to various ER stress-inducing agents, including 10 mM 2-deoxy-D-glucose for 18 h, 10 mM glucosamine for 18 h, 10 μg/ml BFA for 2 h followed by a BFA-free incu-

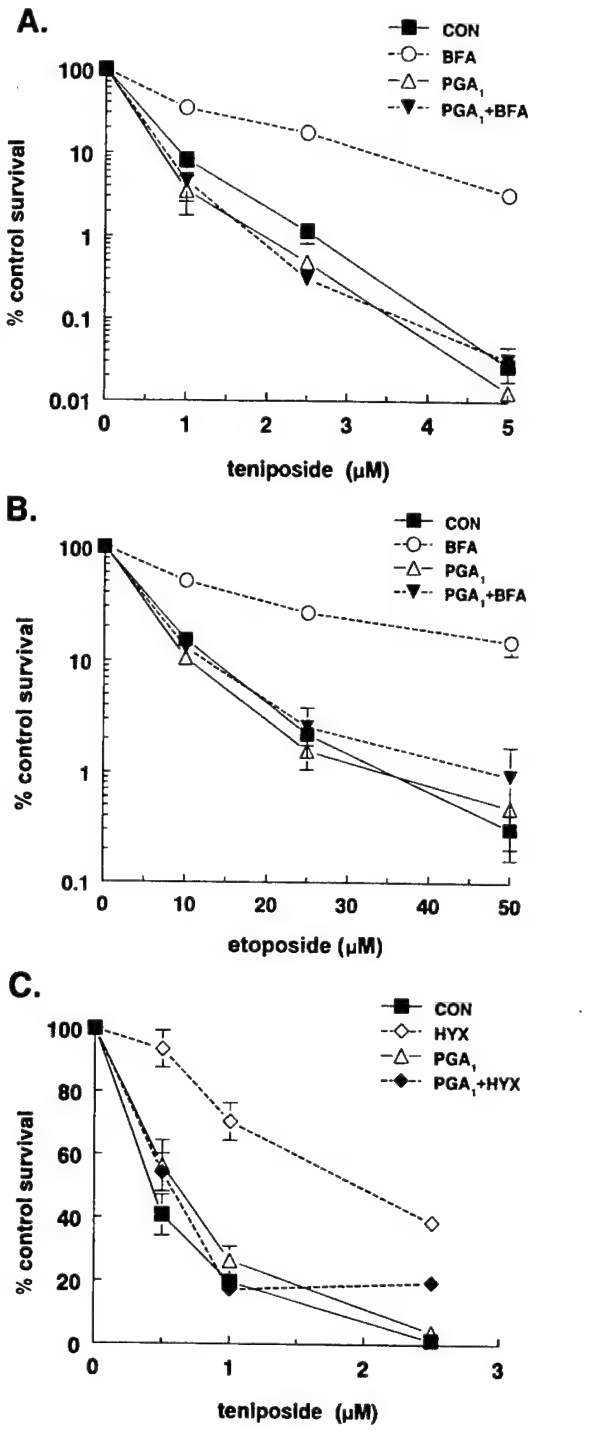


Figure 1. PGA_1 reverses BFA- and HYX-induced resistance to topoII inhibitors. EMT6 cells were treated with 25 μM prostaglandin A_1 (PGA_1) 30 min prior to treatment with 10 μg/ml brefeldin A (BFA) for 2 h followed by 6 h in BFA-free medium (A, B) or hypoxia (HYX) for 8 h (C). Teniposide (A, C) or etoposide (B) was added during the final hour of stress and PGA_1 treatment prior to analysis by colony-forming assay. The toxicity of teniposide (A, C) or etoposide (B) treatment in the absence of stress (CON) is also shown. Values represent the mean percent control cell survival calculated from 3–5 independent experiments with three replicates per experiment; bars are SEM.

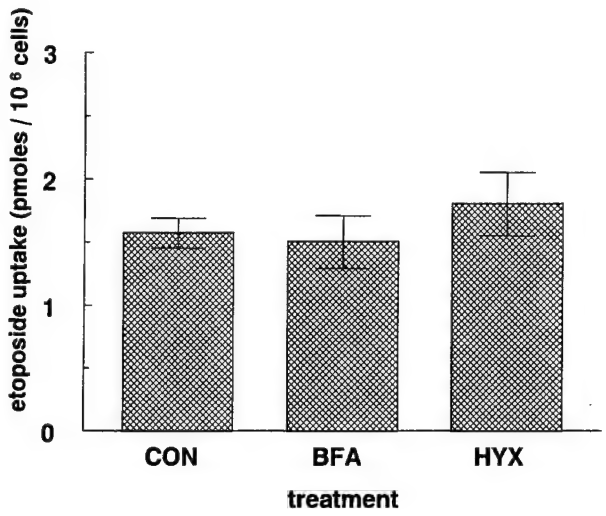


Figure 2. BFA or HYX treatment do not alter cellular etoposide uptake. The uptake of ^{3}H-labeled etoposide was measured in nontreated (CON) or stress-treated cells. EMT6 cells were treated with either 10 μg/ml brefeldin A (BFA) for 2 h followed by 6 h in BFA-free medium or hypoxia (HYX) for 8 h. During the final hour of stress treatment, ^{3}H-labeled etoposide (0.5 μCi/ml, 5.5 nM) and 0.1 μCi ^{14}C-labeled inulin in 50 μM etoposide were added to the cells. Following drug exposure, cells were lysed and analyzed by scintillation spectrometry. Etoposide uptake was determined from the number of counts of radiolabeled etoposide incorporated and converted to picomoles of etoposide per 10^6 cells (pmol/10^6 cells). Values represent the mean drug uptake from three independent experiments with three replicates per experiment; bars are SEM.

bation for 6 h, 5 μg/ml tunicamycin for 6 h, or hypoxia for 18 h. Whole cell and nuclear protein extracts were collected from stress-treated cells and analyzed by Western blot using an anti-topoIIα p170 polyclonal antibody. Figure 3 shows that stress treatment results in no significant alteration in topoII levels from either whole cell (Fig. 3A) or nuclear extracts (Fig. 3B). If anything, increases in whole cell and nuclear topoII were observed. Whole cell extracts from quiescent cells in plateau growth had low levels of topoII (Fig. 3A), as others have observed (35). These data show that stress treatments known to cause resistance to topoII inhibitors do not cause the depletion of total or nuclear topoII in the EMT6 cell line.

To determine whether stressors or drug reversal agents alter topoII activity directly, an in vitro topoII decatenation assay was performed. Purified human topoIIα was incubated with coiled kDNA in the presence of BFA, PGA_1, or the topoII inhibitor teniposide. The resulting DNA samples were separated by electrophoresis and the percent decatenation was determined for each treatment. Figure 4 shows that relatively high concentrations of BFA and PGA_1 (10× those used in cell survival assays) did not inhibit topoII-mediated decatenation of kDNA. However, teniposide treatment resulted in a significant inhibition of topoII-mediated kDNA decatenation (Fig. 4). This suggests that neither stress nor PGA_1 treatment changes the functional activity of topoII. Taken together, Figures 3 and 4 imply that alterations in target enzyme concentration or activity do not explain stress-induced drug resistance or its reversal.

Recent studies have shown that several ER stress-inducing agents activate the transcription factor NF-κB (11,12). Our previous time course data show that the chemical stress agents BFA or the phosphatase inhibitor OA cause marked elevation of NF-κB activation by EMSA. These studies suggest maximal stress-induced NF-κB activation occurs after 4 h of BFA treatment or 8 h of OA treatment (11). Here the time dependence of stress-induced NF-κB activation using the physiological stress hypoxia was determined. Following treatment with hypoxia for 1 to 8 h, nuclear extracts were collected from EMT6 cells, incubated with a radiolabeled NF-κB consensus oligonucleotide, and analyzed by gel electrophoresis. Figure 5 shows that hypoxia treatment results in greatly enhanced levels of free nuclear NF-κB, with a maximal response occurring at the 2-h time point. In our data, two bands of specific binding are detectable that others have suggested are the p65/p50 (upper band) and p50/p50 isoforms of NF-κB (36).

To determine if PGA_1 treatment can inhibit maximal stress-induced NF-κB activation, we analyzed by EMSA nuclear extracts from EMT6 cells treated with 25 μM PGA_1 30 min prior to treatment with either 10 μg/ml BFA for 2 h followed by 2 h in BFA-free medium, hypoxia for 2 h, or 60 nM OA for 8 h (time points of maximal NF-κB activation). Figure 6 shows that BFA (Fig. 6A), hypoxia (Fig. 6B), or OA (Fig. 6C) treatment cause greatly enhanced levels of free nuclear NF-κB. However, PGA_1 pretreatment dramatically inhibited either BFA-, hypoxia-, or OA-induced NF-κB activation (left panel, Fig. 6, A-C, respectively). The specificity of NF-κB binding was assessed with the addition of a 50-fold excess of nonlabeled NF-κB consensus sequence that effectively blocked specific interaction of BFA-, hypoxia-, or OA-induced free nuclear NF-κB with the radiolabeled probe (right panel of Fig. 6A–C, respectively). The addition of a 50-fold excess of AP-1 oligonucleotide resulted in no change in binding of stress-induced NF-κB to the labeled probe (right panel of Fig. 6A–C). Taken together, the data demonstrate that stress induces NF-κB activation that is blocked by PGA_1 pretreatment.

To determine the effects of stress and PGA_1 on the functional activity of NF-κB, we transiently transfected EMT6 cells with a NF-κB-sensitive luciferase reporter construct (pTK-6κB-*Luc*) to measure NF-κB transactivation. This reporter plasmid contains a luciferase gene downstream of a minimal thymidine kinase (TK) promoter with six NF-κB binding sites (27). Following transfection, cells were treated with either 10 μg/ml BFA for 2 h followed by 2 h in BFA-free medium or hypoxia for 2 h (time points of maximal NF-κB activation). Figure 7 shows that cells treated with BFA or hypoxia have enhanced luciferase activity when compared with nonstressed cells. Treatment with 60 nM OA for 8 h resulted in an even greater elevation of luciferase activity. To determine the effects of the reversal agent

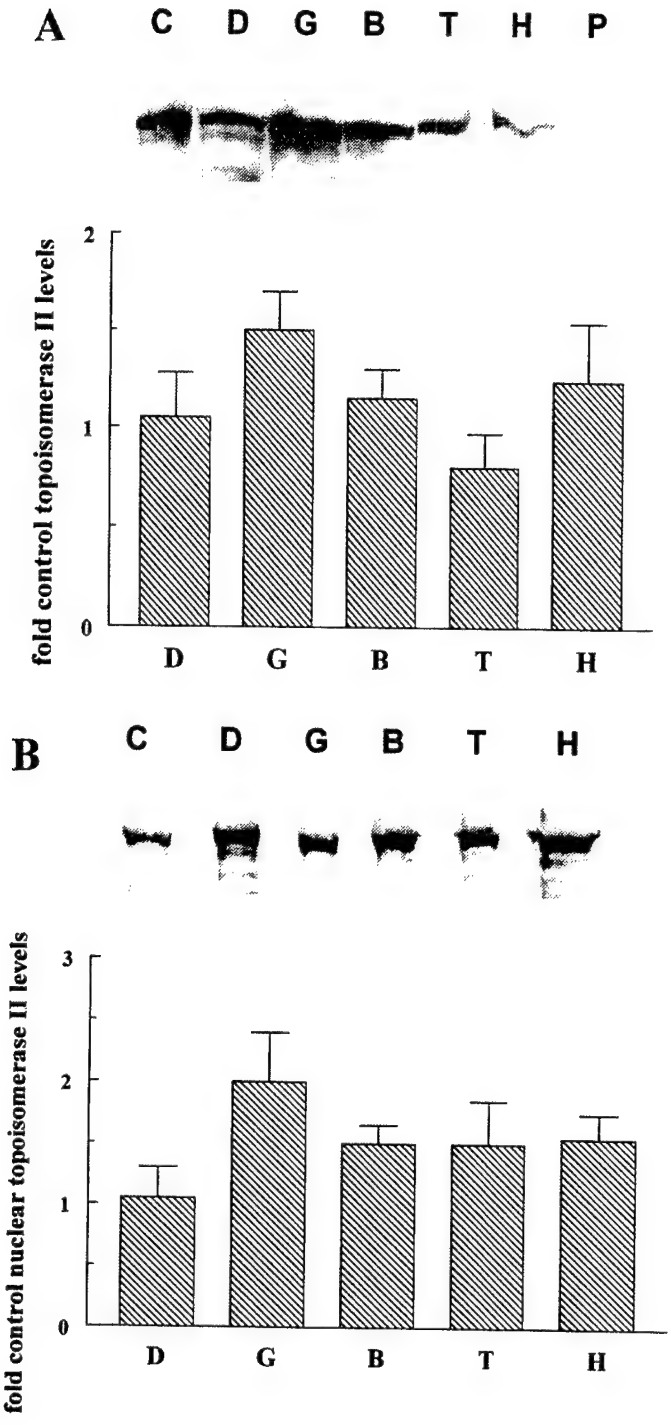


Figure 3. Stress treatment does not alter topoII protein levels. EMT6 cells were exposed to 10 mM 2-deoxy-D-glucose (D) for 18 h, 10 mM glucosamine (G) for 18 h, 10 μg/ml brefeldin A (B) for 2 h followed by 6 h in BFA-free medium, 5 μg/ml tunicamycin (T) for 6 h, or hypoxia (H) for 18 h. Whole cell (A) and nuclear (B) protein samples from nonstressed cells (C), stress-treated cells, and quiescent cells in plateau growth (P) were collected. TopoII protein was detected by Western blot analysis using a primary anti-p170 topoIIα antibody. Shown is a representative blot from one of three independent experiments (upper panel) and the fold control change in topoII levels quantitated by scanning laser denistometry (lower panel); bars are SEM.

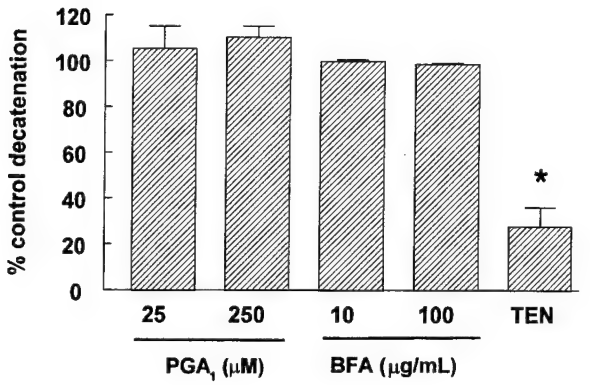


Figure 4. BFA and PGA_1 treatments do not alter the function of human topoII in vitro. Reaction mixtures of 2 units of purified human topoIIα enzyme, 200 ng kDNA, and either 25 or 250 μM prostaglandin A_1 (PGA_1) or 10 or 100 μg/ml brefeldin A (BFA) were incubated at 37°C for 1 h. As a positive control, enzyme and DNA were incubated with 100 μM teniposide (TEN). Decatenation products were separated on an agarose gel with ethidium bromide and quantitated using a Stratagene Eagle Eye transilluminator. Values represent the mean percent control decatenation ± SEM from three independent experiments. *A statistically significant decrease in decatenation was observed with teniposide treatment when compared with control (one-way ANOVA followed by multiple comparison using Bonferroni's test with $P < 0.05$).

PGA_1 on NF-κB transactivation, we treated cells with PGA_1 30 min prior to stress treatment. Our results show that pretreatment with PGA_1 inhibits basal levels of NF-κB transactivation, as well as BFA-, hypoxia-, and OA-induced NF-κB activation (Fig. 7).

Figure 1 shows that cells treated with BFA are resistant to etoposide and teniposide. This duration of BFA treatment was selected because it results in no alterations in cell survival or DNA, RNA, or protein synthesis [(11), data not shown]. To determine if shorter BFA exposures result in similar resistance to teniposide, we treated cells with 10 μg/ml BFA for 30 min followed by a BFA-free recovery for 7.5 h. Teniposide was added during the final hour of BFA treatment prior to analysis by colony-forming assay. Figure 8 shows that a 30-min exposure to BFA results in a level of resistance to teniposide not significantly different from the standard BFA treatment for 2 h.

PGA_1 can prevent the activation of NF-κB and reverse both BFA- and hypoxia-induced resistance to teniposide (Figs. 1, 6, and 7). In these experiments, PGA_1 was added prior to the introduction of stress. In vivo, however, tumors are likely exposed to stress before, during, and after chemotherapy is initiated. An agent that could reverse drug resistance after stress has occurred would be more useful therapeutically. We determined

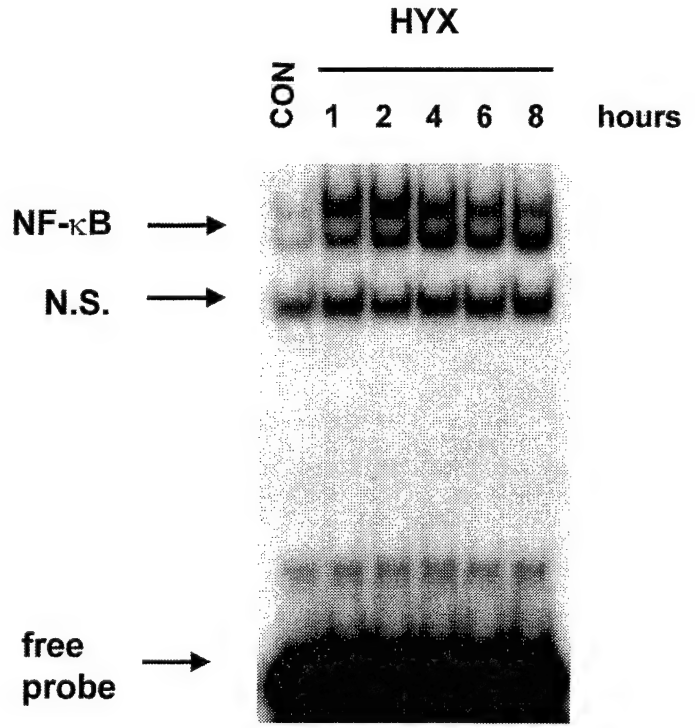
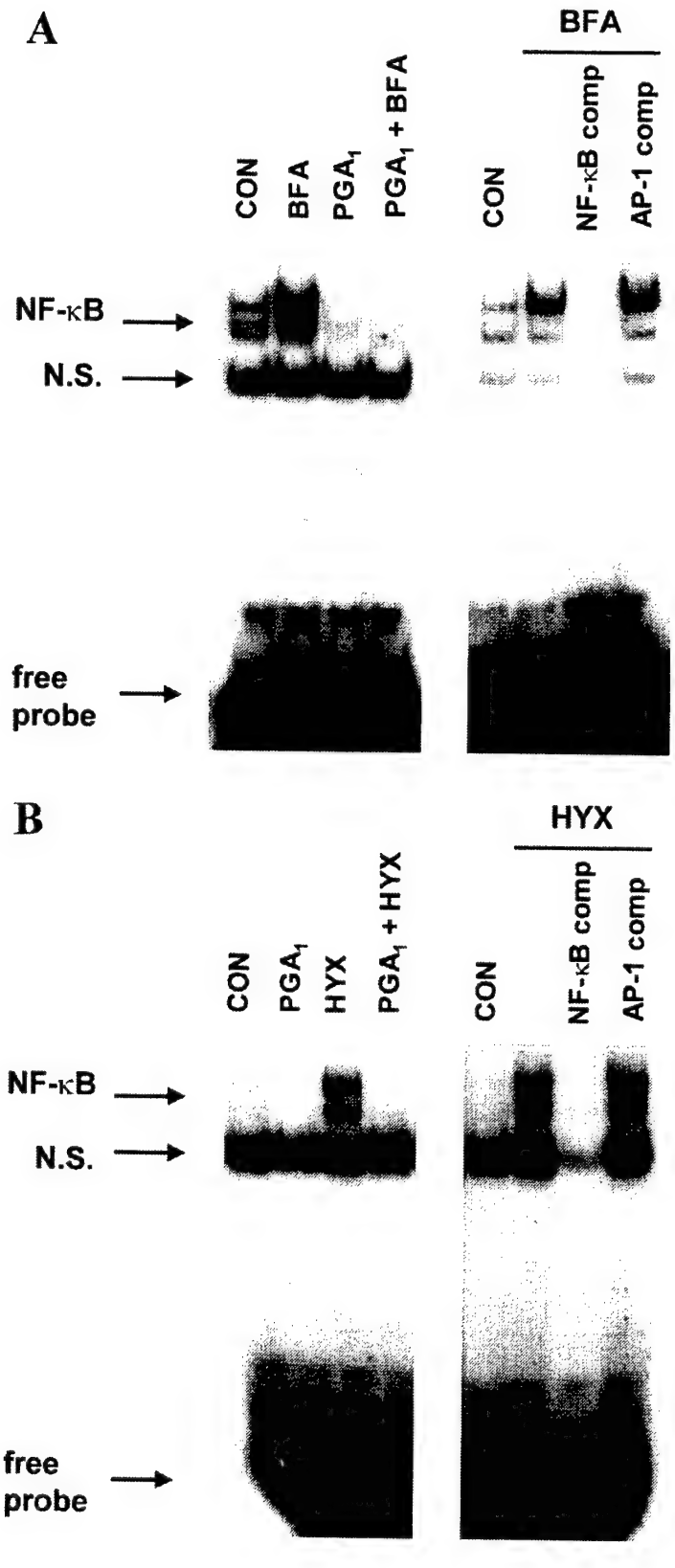


Figure 5. Time course NF-κB activation by HYX stress. EMT6 cells were exposed to hypoxic conditions for 1, 2, 4, 6, or 8 h as described in Materials and Methods. Following treatment, nuclear extracts from nontreated (CON) or hypoxia-treated (HYX) cells were harvested and analyzed by EMSA using a ^{32}P-labeled NF-κB oligonucleotide. Specific binding of NF-κB to the probe (NF-κB), nonspecific binding (N.S.), and unbound probe (free probe) bands are indicated.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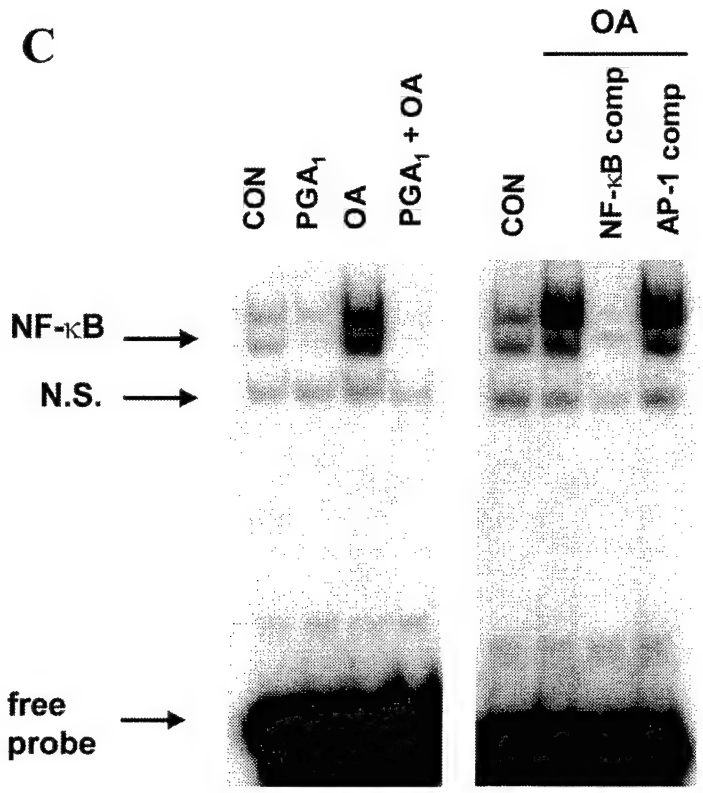

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Figure 6. PGA_1 inhibits stress-induced NF-κB activation. EMT6 cells were treated with 25 μM prostaglandin A_1 (PGA_1) 30 min prior to stress treatment with 10 μg/ml brefeldin A (BFA) (A) for 2 h followed by 2 h in BFA-free medium, hypoxia (HYX) (B) for 2 h, or 60 nM okadaic acid (OA) (C) for 8 h. Following treatment, nuclear extracts were harvested and analyzed by EMSA using a ^{32}P-labeled NF-κB oligonucleotide. Specific binding of NF-κB to the probe (NF-κB), non-specific binding (N.S.), and unbound probe (free probe) bands are indicated. For each experiment, constitutive activation of NF-κB from nontreated cells is also shown (CON). The specificity of NF-κB binding by competition assay was also determined (right panel, A–C). Nuclear extracts from BFA-, HYX-, or OA-treated cells were incubated with oligonucleotide in the presence of a 50-fold excess of either unlabeled NF-κB or AP-1 oligonucleotide (NF-κB comp and AP-1 comp, respectively). Shown in each panel is one representative gel from three independent experiments.

whether PGA_1 could prevent drug resistance when used during various phases of the stress response. Cells were exposed to 10 μg/ml BFA for 30 min followed by a BFA-free recovery for 7.5 h. PGA_1 (25 μM) was added either 30 min prior to BFA treatment, at the same time as BFA treatment, or 30 min, 1, 2, 4, or 6 h after BFA treatment. Teniposide was added during the last hour of stress and PGA_1 treatment prior to analysis by colony-forming assay. In addition, PGA_1 was added 7 h after BFA treatment (and therefore at the same time teniposide was added), 8 h after BFA treatment (1 h after teniposide, immediately before colony-forming assay), or 9 h after BFA treatment (teniposide was removed, PGA_1 was added for 1 h before colony-forming assay). Figure 9 shows that PGA_1 treatment results in a partial but significant reversal of resistance to teniposide even when given up to 7 h after BFA treatment. Treatment with PGA_1 8–9 h after BFA and teniposide treatment failed to inhibit the development of resistance.

DISCUSSION

Solid tumors have a unique physiology that includes the presence of hypoxic and/or glucose-deprived cell subpopulations (6). Hypoxia has been repeatedly shown to limit the responsiveness of tumor cells to ionizing radiation and chemotherapy (7,8). The mechanism by which hypoxic cells develop resistance to radiation and chemotherapy may involve low oxygen tension and poor drug penetration into solid tumors (37,38). Genetic and biochemical alterations that develop in hypoxia-treated cells may also contribute to development of drug resistance, especially towards cell cycle-specific chemotherapeutic agents (39,40). Hypoxia causes changes in genomic stability through the induction of anoxia-responsive endonucleases, which result in DNA breakage and altered gene expression (39). Drug insensitivity may also be explained by the influence of hypoxia on cell cycle arrest and distribution through modulation of the p53, p21, and p27 proteins (40).

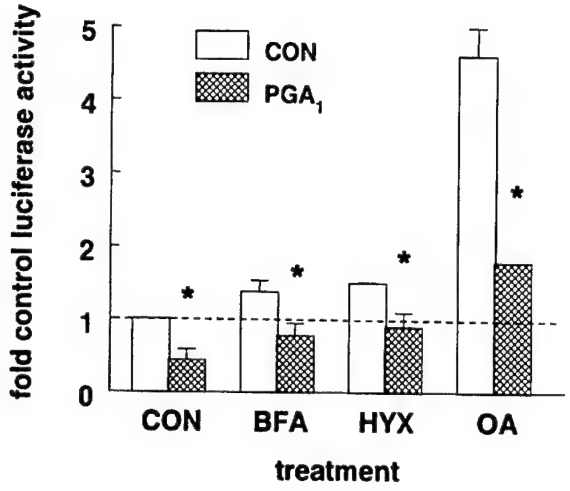

Figure 7. PGA_1 prevents stress-induced NF-κB transactivation. EMT6 cells were transiently transfected with a NF-κB-sensitive luciferase reporter gene. After transfection, cells were treated with 25 μM prostaglandin A_1 (PGA_1) 30 min prior to stress treatment with 10 μg/ml brefeldin A (BFA) for 2 h followed by 2 h in BFA-free medium, hypoxia (HYX) for 2 h, or 60 nM okadaic acid (OA) for 8 h (time points of maximal NF-κB activation). Whole cell lysates from nontreated (CON) and stress-treated cells were assayed for luciferase expression by measuring the fluorescence intensity in the presence of luciferol substrate with scintillation spectroscopy. The fold control luciferase activity was determined by dividing the activity for each treatment by the activity of nontreated cells. Values represent the mean fold control luciferase activity ± SEM from 3–5 independent experiments. *A statistically significant decrease in luciferase activity was observed in nontreated and stress-treated cell extracts from cells pretreated with PGA_1 (one-way ANOVA followed by multiple comparison using Bonferroni's test with $P < 0.05$).

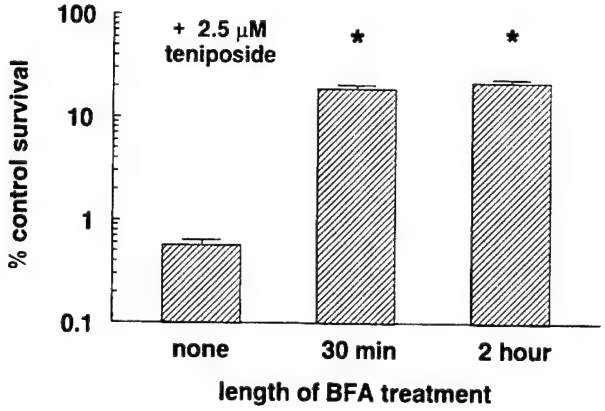

Figure 8. Effect of 30-min BFA exposure on resistance to teniposide. EMT6 cells were exposed to 10 μg/ml brefeldin A (BFA) for either 30 min followed by an incubation in BFA-free medium for 7.5 h (30 min) or for 2 h followed by an incubation in BFA-free medium for 6 h (2 h). During the last hour of BFA treatment, 2.5 μM teniposide was added prior to analysis by colony-forming assay. The toxicity of teniposide in the absence of stress (none) is also shown. Percent control survival was determined for each treatment by dividing the percent survival of BFA-treated cells by the percent survival of solvent-treated cells. Shown are the mean percent control cell survival ± SEM from 3–5 independent experiments with three replicates per experiment. *A statistically significant increase in cell survival was observed in BFA-treated cells compared with nonstressed cells (one-way ANOVA followed by multiple comparison using Bonferroni's test with $P < 0.05$); there was no statistically significant difference in cell survival between the 30-min and 2-h BFA exposures.

Recent data suggest the activation of stress responses by hypoxia may also mediate intrinsic tumor drug resistance. Hypoxic conditions induce cellular glucose-regulated stress proteins, heat shock proteins, and stress-activated protein kinases (41–43). Hypoxia also causes the activation of various stress- and redox-sensitive proteins, such as NF-κB, AP-1, Ref-1 and HIF-1 (18,44,45). The direct effects of these proteins on apoptosis and downstream gene expression provide another explanation for how adverse tumor microenvironmental conditions lead to chemotherapeutic drug resistance.

In this report we show that treatment with the chemical ER stress agent, BFA, or the physiologic stress, hypoxia, induces similar levels of resistance to teniposide. These findings imply that the mechanism of hypoxia-induced resistance may be through the induction of ER stress pathways. Data now suggest there are two major ER-mediated stress response pathways. One pathway, known as the unfolded protein response (UPR), is activated by the presence of abnormally folded proteins in the ER and is characterized by the induction of the glucose-regulated protein GRP78 (19). The second pathway is known as the ER overload pathway (EOR). In this pathway, high levels of protein in the ER cause the release of intracellular calcium stores and activation of the transcription factor NF-κB (19). Our previous data suggest the EOR pathway mediates stress-induced resistance to topoII inhibitors. We have shown that selective activation of the EOR pathway with OA is sufficient to cause the development of resistance to teniposide (11). However, selective activation of the UPR pathway with the glucosidase inhibitor castanospermine results in no change in sensitivity to teniposide (11). Hence, the mechanism of stress-induced resistance to topoII inhibitors may be through stimulation of the EOR pathway and NF-κB activation.

To determine the association between NF-κB activation and the development of resistance to topoII inhibitors, PGA_1 was used. PGA_1 is known to prevent the activation of NF-κB by inhibiting the phosphorylation and upregulation of the inhibitory IκB protein (28,29). Here we demonstrate that PGA_1 treatment effectively blocks NF-κB activation by BFA, hypoxia, or OA treatment. Importantly, PGA_1 prevents BFA- and hypoxia-induced resistance to etoposide and teniposide. These data imply that NF-κB activation through the EOR pathway may mediate both chemical- and physiologic-induced resistance to topoII inhibitors. The findings presented here further substantiate our previous work with the proteasome inhibitor MG-132 (11). Like PGA_1, MG-132 pretreatment blocks BFA-induced NF-κB activation and re-

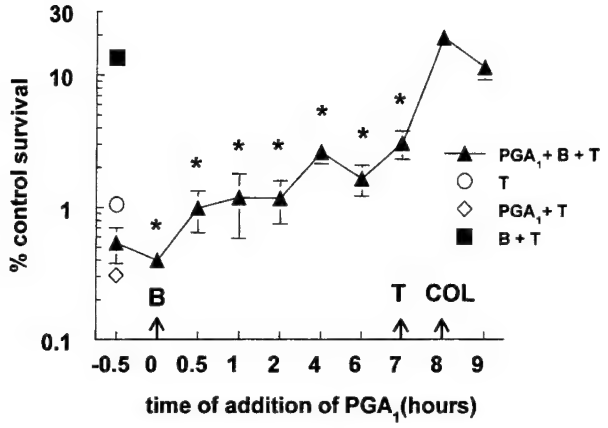

Figure 9. BFA-induced resistance to teniposide can be reversed with PGA_1 after the induction of stress. EMT6 cells were exposed to 10 μg/ml brefeldin A (B) for 30 min (added at the 0-h time point) and then incubated in BFA-free medium for 7.5 h. During the last hour of stress treatment, 2.5 μM teniposide (T) was added (at the 7-h time point) prior to analysis by colony-forming assay (COL, at 8-h time point). To determine the time dependence of reversal, 25 μM prostaglandin A_1 (PGA_1) was added either 30 min before BFA treatment (at the –0.5-h time point) or after BFA treatment (at the 0.5-, 1-, 2-, 4-, or 6-h time points). Percent control survival was determined by dividing the cell survival of BFA-treated cells by the cell survival of nontreated cells. Values represent the mean percent control cell survival ± SEM from three independent experiments with three replicates per experiment. *A statistically significant decrease in cell survival was observed in cells treated with PGA_1 prior to, during, and up to 7 h after BFA treatment when compared with BFA treatment alone (one-way ANOVA followed by multiple comparison using Bonferroni's test with $P < 0.05$).

verses BFA-induced resistance to teniposide (11). The fact that MG-132 and PGA_1 inhibit NF-κB activation by different mechanisms and both reverse drug resistance further strengthens the role of NF-κB activation in stress-induced drug resistance.

In the present study we investigated whether BFA- or hypoxia-induced resistance or its reversal with PGA_1 could be explained by the more common mechanisms of drug resistance. Resistance to topoII inhibitors is often explained by alterations in drug accumulation, topoII levels, and/or topoII activity. Our data demonstrate, however, that the accumulation of etoposide was not altered by either chemical or physiologic stress treatment. Similarly, analyses of whole cell and nuclear protein extracts show that topoII protein levels or in vitro topoII activity were not altered by chemical or physiologic stress. Others have suggested that PGA_1 treatment inhibits topoII ($IC_{50} = 98$ μM) (46), but our data show concentrations of PGA_1 up to 250 μM did not alter topoII-mediated kDNA decatenation. It is therefore unlikely that stress-induced resistance to topoII inhibitors is caused by changes in drug accumulation by the P-glycoprotein or MRP pumps or by altered topoII levels or activity. Moreover, the reversal of stress-induced resistance with PGA_1 cannot be explained by decreased topoII activity.

EMT6 cells exposed to BFA for only 30 min developed resistance to teniposide. This finding suggests that short periods of physiological stress can cause drug resistance. Consequently, even transient losses of blood flow to tumors may have a dramatic impact on whether a tumor will respond to chemotherapeutic treatment. In the present study, cells were exposed to PGA_1 after BFA treatment to determine if drug resistance could be reversed following the induction of stress. PGA_1 pretreatment partially reversed BFA-induced resistance to teniposide when given up to 7 h after stress treatment. These data suggest that PGA_1 is an effective reversal agent not only when given before stress treatment, but even when given several hours after stress treatment. This implies that the intrinsic drug resistance of solid tumors can be reversed during or after the induction of stress. This finding is especially significant because it is likely that most solid tumors have been or are currently exposed to stress prior to chemotherapy treatment.

It is well known that solid tumors contain stressful microenvironments and are intrinsically resistant and insensitive to cancer chemotherapeutics. Recent data show GRP78 levels are elevated in malignant breast lesions when compared with nonmalignant breast lesions (47). Moreover, the concentrations of etoposide and teniposide we have used lie within or below the ranges of that achieved clinically (48,49). Therefore, the form of drug resistance characterized here that is induced by physiological and chemical stressors likely exists in solid tumors and may explain in part the intrinsic drug resistance of tumors seen clinically. Our data further suggest that the coadministration of agents that inhibit NF-κB activation would enhance the efficacy of topoII inhibitors in the treatment of cancer. Others have reported that inhibition of NF-κB activation enhances the toxicity of anticancer agents both in vivo and in vitro. Inhibition of NF-κB activation with proteasome inhibitors increases the toxicity of chemotherapeutics in in vivo tumor assays (50,51). Expression of a mutant IκBα protein to inhibit NF-κB activation enhances the sensitivity of tumor cells to apoptotic death by tumor necrosis factor, paclitaxel, and daunorubicin (22,24). In addition, inducible expression of mutant IκBα sensitizes physiologic or chemically stress-treated EMT6 cells to etoposide (52). Mutant IκBα expression in vivo reduces growth of head and neck squamous cell carcinoma (53) and sensitizes chemoresistant tumors to the toxic effects of camptothecin (23). The results from experiments with the phosphorylation site-deficient IκBα mutants demonstrate that NF-κB activation plays a prominent role in mediating sensitivity of cells to chemotherapeutic agents (22–24,52,53). Taken together, our data suggest that PGA_1 may represent a new class of antitumor agents that improve the clinical efficacy of topoII-directed agents by inhibiting the NF-κB-mediated stress responses that limit the effectiveness of cancer chemotherapeutics.

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Tampa, for providing the primary topoIIα antibody. We also thank Dr. L. K. Barrows and his laboratory from the University of Utah, Salt Lake City, for their helpful advice regarding the in vitro topoII assay. Parts of this work were submitted in partial fulfillment of the requirements for the Master's of Science degree (Y.C.B.) and the Doctor of Philosophy degree (R.L.R. and Z.P.L.) and will be submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree (L.M.B.), Department of Pharmacology, Institute for Biomedical Sciences of the Columbian College of Arts and Sciences, The George Washington University. This research was supported in part by Army Breast Cancer Initiative Award #99-1-9186 and by a faculty research enhancement award from GWUMC (K.A.K.).

REFERENCES

1. Beck, W. T.; Danks, M. K. Mechanisms of resistance to drugs that inhibit DNA topoisomerases. Semin. Cancer Biol. 2(4):235–244; 1991.
2. Endicott, J. A.; Ling, V. The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu. Rev. Biochem. 58:137–171; 1989.
3. Cole, S. P.; Bhardwaj, G.; Gerlach, J. H.; Mackie, J. E.; Grant, C. E.; Almquist, K. C.; Stewart, A. J.; Kurz, E. U.; Duncan, A. M.; Deeley, R. G. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 258: 1650–1654; 1992.
4. Danks, M. K.; Schmidt, C. A.; Dirtain, M. C.; Suttle, D. P.; Beck, W. T. Altered catalytic activity of and DNA cleavage by DNA topoisomerase II from human leukemic cell selected for resistance to VM-26. Biochemistry 27:8861–8869; 1988.
5. Sutherland, R.; Freyer, J.; Mueller-Klieser, W.; Wilson, R.; Heacock, C.; Sciandra, J.; Sordat, B. Cellular growth and metabolic adaptations to nutrient stress environments in tumor microregions. Int. J. Radiat. Oncol. 12:611–615; 1986.
6. Vaupel, P.; Kallinowski, F.; Okunieff, P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumor: A review. Cancer Res. 49:6449–6465; 1989.
7. Teicher, B. A.; Holden, S. A.; al-Achi, A; Herman, T. S. Classification of antineoplastic treatments by their differential toxicity toward putative oxygenated and hypoxic tumor subpopulations in vivo in the FSaIIC murine fibrosarcoma. Cancer Res. 50:3339–3344; 1990.
8. Sakata, K.; Kwok, T. T.; Murphy, B. J.; Laderoute, K. R.; Gordon, G. R; Sutherland, R. M. Hypoxia-induced drug resistance: Comparison to P-glycoprotein-associated drug resistance. Br. J. Cancer 64:809–814; 1991.
9. Shen, J.; Hughes, C.; Chao, C.; Cai, J.; Bartels, C.; Gessner, T.; Subjeck, J. Conduction of glucose-regulated proteins and doxorubicin resistance in Chinese hamster cells. Proc. Natl. Acad. Sci. USA 84:3278–3282; 1987.
10. Hughes, C.; Shen, J.; Subjeck, C. Resistance to etoposide induced by three glucose-regulated stresses in Chinese hamster ovary cells. Cancer Res. 49:4452–4454; 1989.
11. Lin, Z.; Boller, Y.; Amer, S.; Russell, R.; Pacelli, K.; Patierno, S.; Kennedy, K. Prevention of brefeldin A-induced resistance to teniposide by the proteasome inhibitor MG-132: Involvement of NF-κB activation in drug resistance. Cancer Res. 58:3059–3065; 1998.
12. Koong, A. C.; Auger, E. A.; Chen, E. Y.; Giaccia, A. J. The regulation of GRP78 and messenger RNA by hypoxia is modulated by protein kinase C activators and inhibitors. Radiat. Res. 138(Suppl. 1):S60–S63; 1994.
13. Miyake, H.; Hara, I.; Arkawa, S.; Kamidono, S. Stress protein GRP78 prevents apoptosis induced by calcium ionophore, ionomycin, but not by glycosylation inhibitor, tunicamycin, in human prostate cancer cells. J. Cell Biochem. 77(3):396–408; 2000.
14. Urban, M. B.; Schreck, R.; Baeuerle, P. A. NF-κB contacts DNA by a heterodimer of the p50 and p65 subunits. EMBO J. 10:1817–1825; 1991.
15. Ghosh, S.; Baltimore, D. Activation in vitro of NF-κB by phosphorylation of its inhibitor IκB. Nature 344:678–682; 1990.
16. Palombella, V.; Arndo, O.; Goldberg, A.; Maniatis, T. The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. Cell 78:773–785; 1994.
17. Lenardo, M.; Baltimore, D. NF-κB: A pleiotropic mediator of inducible and tissue-specific gene control. Cell 58:227–229; 1989.
18. Koong, A. C.; Chen, E. Y.; Giaccia, A. J. Hypoxia cause the activation of nuclear factor-kappaB through the phosphorylation of IkappaBalpha on tyrosine residues. Cancer Res. 54:1425–1430; 1994.
19. Pahl, H.; Baeuerle, P. The ER-overload response: Activation of NF-κB. Trends Biochem. Sci. 22:63–67; 1997.
20. Wang, C. Y.; Mayo, M. W.; Korneluk, R. G.; Goeddel, D. V.; Baldwin, A. S., Jr. NF-kappaB and antiapoptosis: Induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 281:1680–1683; 1998.
21. Wang, C. Y.; Guttridge, D. C.; Mayo, M. W.; Baldwin, A. S., Jr. NF-kappaB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. Mol. Cell. Biol. 19(9):5923–5929; 1999.
22. Batra, R. K.; Guttridge, D. C.; Brenner, D. A.; Dubinett, S. M.; Baldwin, A. S.; Boucher, R. C. IkappaBalpha gene transfer is cytotoxic to squamous-cell lung cancer cells and sensitizes them to tumor necrosis factor-alpha-mediated cell death. Am. J. Respir. Cell. Mol. Biol. 21(2):238–245; 1999.
23. Cusack, J. C., Jr.; Liu, R.; Baldwin, A. S., Jr. Inducible chemoresistance to 7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin (CPT-11) in colorectal cancer cells and a xenograft model is overcome by inhibition of nuclear factor-kappaB activation. Cancer Res. 60(9):2323–2330; 2000.
24. Huang, Y.; Johnson, K. R.; Norris, J. S.; Fan, W. Nuclear factor-kappaB/IkappaB signaling pathway may contribute to the mediation of paclitaxel-induced apoptosis in solid tumor cells. Cancer Res. 60(16):4426–4432; 2000.
25. Lippincott-Schwartz, J.; Yuan, L.; Tipper, C.; Amherdt, M.; Orci, L.; Klausner, R. D. Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. Cell 67(3):601–616; 1991.
26. Kato, T.; Fukushima, M.; Kurozumi, S.; Noyori, R. Antitumor activity of Δ7-prostaglandin A1 and Δ12-prostaglandin j2 in vitro and in vivo. Cancer Res. 46:3538–3542; 1986.
27. Carattoli, A.; Fortini, D.; Rozera, C.; Giorgi, C. Inhibition of HIV-1 transcription by cyclopentenone prostaglandin A1 in jurkat T lymphocytes. J. Biol. Regul. Homeost. Agents 14(3):209–216; 2000.
28. Thomas, S. C.; Ryan, M. A.; Shanley, T. P.; Wong, H. R. Induction of the stress response with prostaglandin-induced I-kappaBalpha gene expression. FASEB J. 12(13):1371–1378; 1998.
29. Rossi, A.; Kaphai, P.; Natoli, G.; Takahashi, T.; Chen, Y.; Karin, M.; Santoro, M. G. Anti-inflammatory cyclopentenone prostaglandin inhibitors of IkappaB kinase. Nature 403(6765):103–108; 2000.
30. Rockwell, S.; Kennedy, K. A.; Sartorelli, A. C. Mitomycin-C as a prototype bioreductive alkylating agent: In vitro studies of metabolism and cytotoxicity. Int. J. Radiat. Oncol. Biol. Phys. 8(3–4):753–755; 1982.
31. Sullivan, D. M.; Latham, M. D.; Ross, W. E. Proliferation-dependent topoisomerase II content as a determinant of antineoplastic drug action in human, mouse, and Chinese hamster ovary cells. Cancer Res. 47:3973–3979; 1987.
32. Scheuren, N.; Bang, H.; Munster, T.; Brune, K.; Pahl, H. Modulation of transcription factor NF-kappaB by enantiomers of the non-steroidal drug ibuprofen. Br. J. Pharmacol. 123(4):645–652; 1998.
33. Motulsky, H. Intuitive biostatistics. New York: Oxford University Press; 1995.
34. Russell, R. L. Alteration of EMT6 cell sensitivity to VM-26 in the presence of the glucose-regulated stress response. Dissertation, 1994.
35. Prosperi, E.; Sala, E.; Negri, C.; Oliani, C.; Supino, R.; Astraldi-Ricotti, G. B.; Bottiroli, G. Topoisomerase II alpha and beta in human tumor cells grown in vitro and in vivo. Anticancer Res. 12(6B):2093–2099; 1992.
36. Contant, K.; Atwood, W. J.; Traub, R.; Tornatore, C.; Major, E. O. An increase in p50/p65 NF-κB binding to the HIV-1 LTR is

not sufficient to increase viral expression in the primary human astrocyte. Virology 205(2):586–590; 1994.
37. Durand, R. E. Distribution and activity of antineoplastic drugs in a tumor model. J. Natl. Cancer Inst. 81:146–152; 1989.
38. Rockwell, S. Oxygen delivery: Implications for the biology and therapy of solid tumors. Oncol. Res. 9:383–390; 1997.
39. Russo, C. A.; Weber, T. K.; Volpe, C. M.; Stoler, D. M.; Petrelli, N. J.; Rodriguez-Bigas, M.; Burhans, W. C.; Anderson, G. R. An anoxia inducible endonuclease and enhanced DNA breakage as contributors to genomic instability in cancer. Cancer Res. 55(5): 1122–1128; 1995.
40. Chandel, N. S.; Vander Heiden, M. G.; Thompson, C. B.; Schumacker, P. T. Redox regulation of p53 during hypoxia. Oncogene 19(34):3840–3848; 2000.
41. Koong, A. C.; Chen, E. Y.; Giaccia, A. J. Increased cytotoxicity of chronic hypoxic cells by molecular inhibition of GRP78 induction. Int. J. Radiat. Oncol. Biol. Phys. 28:661–666; 1994.
42. Patel, B.; Khaliq, A.; Jarvis-Evans, J.; Boulton, M.; Arrol, S.; Mackness, M.; McLeod, D. Hypoxia induces HSP70 gene expression in human hepatoma (HEP G2) cells. Biochem. Mol. Biol. Int. 36(4):907–912; 1995.
43. Conrad, P. W.; Millhorn, D. E.; Beitner-Johnson, D. Hypoxia differentially regulates the mitogen- and stress-activated protein kinases. Role of Ca^{2+}/CaM in the activation of MAPK and p38 gamma. Adv. Exp. Med. Biol. 475:293–302; 2000.
44. Yao, K. S.; Xanthoudakis, S.; Curran, T.; O'Dwyer, P. J. Activation of AP-1 and of a nuclear redox factor, Ref-1, in the response of HT29 colon cancer cells to hypoxia. Mol. Cell Biol. 14(9): 5997–6003; 1994.
45. Carmeliet, P.; Dor, Y.; Herbert, J. M.; Fukumura, D.; Brusselmans, K.; Dewerchin, M.; Neeman, M.; Bono, F.; Abramovitch, R.; Maxwell, P.; Koch, C. J.; Ratcliffe, P.; Moons, L.; Jain, R. K.; Collen, D.; Keshert, E. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation, and tumor angiogenesis. Nature 395(6701):525; 1998.
46. Suzuki, K.; Shono, F.; Uyeda, M. Inhibition of topoisomerases by antitumor prostaglandins. Bioscience 62(10):2073–2075; 1998.
47. Fernandez, P. M.; Tabbara, S. O.; Jacobs, L. K.; Manning, F. C.; Tsangaris, T. N.; Schwartz, A. M.; Kennedy, K. A.; Patierno, S. R. Overexpression of the glucose-regulated stress gene GRP78 in malignant but not benign human breast lesions. Breast Cancer Res. Treat. 59(1):15–26; 2000.
48. Van Tellingen, O.; Boogerd, W.; Nooijen, W. J.; Beijnen, J. H. The vascular compartment hampers accurate determination of teniposide penetration into brain tumor tissue. Cancer Chemother. Pharmacol. 40:330–334; 1997.
49. Chen, C. L.; Uckun, F. M. Highly sensitive liquid chromatography-electorspray mass spectrometry (LC-MS) method for the determination of etoposide levels in human serum and plasma. J. Chromatogr. B. Biomed. Sci. Appl. 744(1):91–98; 2000.
50. Teicher, B. A.; Ara, G.; Herbst, R.; Palombella, V. J.; Adams J. The protease inhibitor PS-341 in cancer therapy. Clin. Cancer Res. 5(9):2638–2645; 1999.
51. Ogiso, Y.; Tomida, A.; Lei, S.; Omura, S.; Tsuruo, T. Proteasome inhibition circumvents solid tumor resistance to topoisomerase II-directed drugs. Cancer Res. 60(9):2429–2434; 2000.
52. Brandes, L. M.; Lin, Z. P.; Patierno, S. R.; Kennedy, K. A. Reversal of physiologic stress-induced resistance to topoisomerase II inhibitors using an inducible phosphorylation site-deficient mutant of IκBα. Mol. Pharm. 60(3):559–567; 2001.
53. Duffey, D. C.; Chen, Z.; Dong, G.; Ondrey, F. G.; Wolf, J. S.; Brown, K.; Siebenlist, U.; Van Waes, C. Expression of a dominant-negative mutant inhibitor-kappaBalpha of nuclear factor-kappaB in human head and neck squamous cell carcinoma inhibits survival, proinflammatory cytokine expression, and tumor growth in vivo. Cancer Res. 59(14):3468–3474; 1999.

EXPRESSION PROFILING REVEALS A ROLE FOR TGF-β AND THE PDGFRα/MAPK SIGNALING PATHWAY IN THE DEVELOPMENT OF STRESS-INDUCED DRUG RESISTANCE

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Abstract

One of the major limiting factors to the successful treatment of breast cancer is the development of drug resistance. Adverse conditions associated with solid tumor progression, which trigger cellular stress responses, may underlie the mechanisms of intrinsic chemotherapeutic drug resistance. Our data show that treatment of EMT6 mouse mammary tumor cells with the chemical stress agent, brefeldin A (BFA) or the physiologic stress, hypoxia induce comparable levels of resistance to the topoisomerase II inhibitor, etoposide. To determine common mechanisms for the development of chemical- and physiologic-induced drug resistance, we have performed expression analysis of stress-treated EMT6 cells using the Affymetrix GeneChip system. Notably, our data show that BFA or hypoxia treatment result in enhanced expression of transforming growth factor-β (TGF-β) and decreased expression of the platelet-derived growth factor receptor, PDGFRα, and the mitogen-activated protein kinase (MAPK), MEK1. Western blot analysis confirms that TGF-β protein levels are enhanced and PDGFRα levels are diminished with BFA or hypoxia treatment. Although total MEK1 and MEK2 levels were not altered by stress treatment, analysis with an antibody selective for phosphorylated forms of MEK1 and MEK2 (phospho-MEK1/2) reveals phospho-MEK1/2 levels are reduced with stress treatment. *In vitro* studies show treatment with TGF-β, inhibition of PDGFRα with blocking antibodies, or inhibition of MEK1/2 with U0126 treatment are sufficient to cause etoposide resistance. Taken together, our results provide evidence for TGF-β activation and subsequent down-regulation of the PDGFRα/MAPK signaling pathway in the development of tumor drug resistance and further suggest that modulation of TGF-β, PDGFRα or the MAPK cascade may enhance the clinical effectiveness of conventional anticancer chemotherapies.

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Materials and Methods

Cell Culture. EMT6 mouse mammary tumor cells (provided by Dr. S. Rockwell, Yale University) were grown in Waymouth's MB 752/1 medium (GibcoBRL, Grand Island, NY) supplemented with L-glutamine (Life Technology, Gaithersburg, MD), 15% fetal bovine serum (Sigma, St. Louis, MO), 100 units/ml streptomycin and 25 μg/ml gentamicin sulfate (Biofluids, Rockville, MD) as described previously (Lin et al., 1998; Boller et al., 2001; Brandes et al., 2001). MDA-MB231 cells were maintained in Eagle's minimum essential medium (Sigma) supplemented with 2.2 g/L sodium bicarbonate (Sigma), 10% fetal bovine serum (Sigma), 0.1 mM non-essential amino acids (Gibco), 0.2 mM L-glutamine (Sigma), 1X antibiotic-antimycotic (Biofluids), 2 mM Hepes buffer (Sigma), 1 mM sodium pyruvate (Sigma).

Reagents and Treatments. Brefeldin A (Sigma) at 10 μg/ml for 2 h followed by a 6 h BFA-free recovery; Hypoxia by exposure of cells to a humidified mixture of 95% N_2, 5% CO_2 (certified O_2<0.05ppm) at 37°C for 8 h; Etoposide (Sigma) at 10-50 μM 1 h prior to colony forming assay. Ponasterone A (Invitrogen, San Diego, CA) at 0.1-10 μM for 24 h; TGF-β (Sigma) at 0.1 or 10 ng/ml for 8 h; blocking anti-PDGFRα antibodies (R&D Systems) at 1 or 10 ng/ml for 8 h; Castanospermine (Sigma) at 10 μg/ml for 6 h; U0126 (Sigma) at 10 or 30 μM for 8 h; SB203580 (Sigma) at 10 μM for 8 h.

Colony forming assay. Following treatment, cells were harvested with trypsin, counted and serially diluted in Waymouth media. Cells were seeded in triplicate tissue culture dishes and incubated under maintenance conditions for 7 d. Colonies were stained with 0.25% crystal violet and counted as previously described (Lin et al., 1998; Boller et al., 2001; Brandes et al., 2001).

Affymetrix GeneChip Expression Analysis. Following drug and stress treatments, total RNA was collected using TRIzol reagent (Sigma) and converted to double-stranded cDNA using the SuperScript Choice System (GibcoBRL). For cDNA cleanup, 162 μl of (25:24:1) phenol:chloroform:isoamyl alcohol saturated with 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (Sigma) was added and the samples were transferred to Phase Lock Gel PLG I-light tubes (Eppendorf, Westbury, NY). After centrifugation at 14,000g for 2 min, the upper aqueous phase was isolated and 0.5 volumes of cold 7.5 M NH_4Ac (Sigma) and 2.5 volumes of absolute ethanol (Sigma) were added. cDNA was pelleted by centrifugation at 14,000g for 20 min at 4°C. The resulting pellets were washed twice with 0.5 ml of cold 80% ethanol, air-dried and resuspended in 12 μl nuclease-free water. Labeled cRNA was synthesized using the ENZO BioArray™ High Yield™ RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY, distributed by Affymetrix, Santa Clara, CA). cRNA samples (20 μg) were fragmented by incubation in 8 μl of 5X fragmentation buffer (200 mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc) at 94°C for 35 min. For hybridization, 15 μg of cRNA was mixed with 5 μl of 3 nM control oligonucleotide B2 (Affymetrix), 15 μl of 20X eukaryotic hybridization controls (Affymetrix), 3 μl of 10 mg/ml Herring sperm DNA (Fisher, Pittsburgh, PA), 3 μl of 50mg/ml acetylated bovine serum albumin (GibcoBRL), and 150 μl of 2X Hybridization Buffer [200 mM MES (Sigma), 2M (Na^+), 40 mM EDTA, 0.02% Tween 20]. Prior to hybridization, cocktails were incubated at 99°C for 5 min and then at 45°C for 5 min. Target RNA quality was first assessed using Test2 Probe Array Chips (Affymetrix). After confirmation on a test chip, 200 μl of hybridization cocktail was incubated on Mu11K gene chips (Affymetrix) for 16 h at 45°C with rotation (60 cycles/min). Following incubation, Mu11K arrays were washed and stained using the Affymetrix GeneChip Fluidics Station 400 using the standard array format EukGE-WS2 protocol according to the manufacturer's recommendations (Affymetrix). In brief, arrays were washed 10 cycles of 2 mixes/cycle with Wash Buffer A [6X SSPE (1 M NaCl, 0.067 M NaH_2PO_4, 6.67 mM EDTA), 0.01% Tween 20, 0.005% Antifoam (Sigma)] at 25°C, washed 4 cycles of 15 mixes/cycle with Wash Buffer B [100 mM MES, 0.1 M (Na^+), 0.01% Tween 20] at 50°C, stained for 10 min in 600 μl of SAPE stain (100 mM MES, 1 M [Na+], 0.05% Tween 20, 0.005% Antifoam, 2 mg/ml acetylated BSA, and 10 μg/ml steptavidin phycoerythrin), washed 10 cycles of 4 mixes/cycle with Wash Buffer A at 25°C, stained for 10 min in 600 μl of antibody solution [100 mM MES, 1M [Na+], 0.05% Tween 20, 0.005% antifoam, 2 mg/ml acetylated BSA (Fisher), 0.1 mg/ml normal goat IgG (Sigma), and 3 μg/ml

biotinylated anti-strepavidin antibody (Sigma)] at 25°C, stained for 10 min in 600 µl of SAPE stain at 25°C, and washed for 15 cycles of 4 mixes/cycle with Wash Buffer A at 30°C. Arrays were scanned twice using an argon-ion laser at a wavelength of 570 nm (Affymetrix).

Western blot analysis. Following drug and stress treatment, cells were washed in cold PBS, lysed in 100 µl of 1X SDS sample buffer (125 mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 0.006% bromophenol blue) and boiled for 5 min. Protein concentration was then determined using the BCA method (Pierce, Rockford, IL). Protein samples (20-100 µg) were mixed with 2X SDS (250 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 0.012% bromophenol blue, 2% β-mercaptoethanol) and boiled for 5 min. Proteins were separated on a 10% SDS-polyacrylamide gel (4% stacking gel, pH 6.8, 10% resolving gel, pH 8.8, 30: 0.8 acrylamide:bisacrylamide) at 120 V for 2 h in a running buffer containing 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The resulting gel was transferred to a nitrocellulose membrane by electrophoresis for 45 min at 55 V in 1X CAPS buffer (10 mM CAPS, pH 11). Following transfer, the membrane was incubated in 1X TBST (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) with 1% bovine serum albumin. Membranes were incubated with primary antibody (Santa Cruz Biotechnology) diluted 1000-fold in 1X TBST with 1% BSA overnight at 4°C, incubated with an HRP-conjugated IgG anti-rabbit secondary antibody (1:10000 dilution in 1X TBST with 1% BSA) for 1 h at room temperature and visualized with enhanced chemiluminescent reagent (Pierce, Rockford, IL).

Statistics. Statistically significant changes in these data were determined using one way ANOVA analysis with multiple comparisons performed using Bonferroni's test at $p<0.05$ (Motulsky, 1995).

Introduction

Physiological stresses associated with solid tumor development play an important role in resistance to chemotherapeutic agents. Cancer cell drug resistance can be reproduced in the laboratory with chemical stress agents that mimic biological stress conditions. Brefeldin A (BFA) induces cellular stress responses, disrupts transport from the endoplasmic reticulum (ER) to the Golgi and causes resistance of breast cancer cells to the topoisomerase II inhibitors (topoII), etoposide and doxorubicin. Our data show the extent of resistance observed with BFA stress is not different than that induced with hypoxia, suggesting that the mechanisms of physiologic stress-induced tumor resistance may be through the activation of cellular stress responses. BFA and hypoxia both cause activation of the nuclear transcription factor NF-κB. We have shown that inhibition of NF-κB activation pharmacologically or with expression of a mutant inhibitory IκBαM protein prevents stress-induced resistance to etoposide. To determine if a common mechanism underlies the development of BFA- and hypoxia-induced drug resistance through NF-κB activation, we expression profiled stress-treated vector-transfected (VCT) and IκBαM-expressing cells using the Affymetrix GeneChip oligonucleotide arrays. After determining which genes were coordinately regulated during both stress responses, we selected candidate genes for further confirmation of protein expression by western blot and studied the role of each in the development of drug resistance *in vitro* by the colony forming assay. Our data suggest a putative mechanism for the development of both chemical and physiologic stress-induced drug resistance through NF-κB activation and for the prevention of stress-induced drug resistance through the inhibition of NF-κB activation. Our data further imply that modulation of NF-κB or the downstream members of the stress responses characterized here may enhance the therapeutic efficacy of conventional clinical cancer chemotherapeutics.

Figure 1. Chemical and physiologic stress induce resistance to etoposide in breast cancer cells. EMT6 cells were treated with 10 μg/mL brefeldin A (BFA) for 2 h followed by a BFA-free recovery for 6 h or with hypoxia (HYX) for 8 h. During the last hour of stress treatment, etoposide (10, 25 or 50 μM for EMT6) was added prior to analysis by colony forming assay. The toxicity of etoposide in the absence of stress (CON) is also shown. Results are the mean percent control cell survival ± SEM from 3-5 independent experiments. *, a statistically significant increase in cell survival was observed in BFA-treated cells compared to CON cells; #, a statistically significant increase in cell survival was observed in HYX-treated cells compared to CON cells ($p<0.05$, ANOVA).

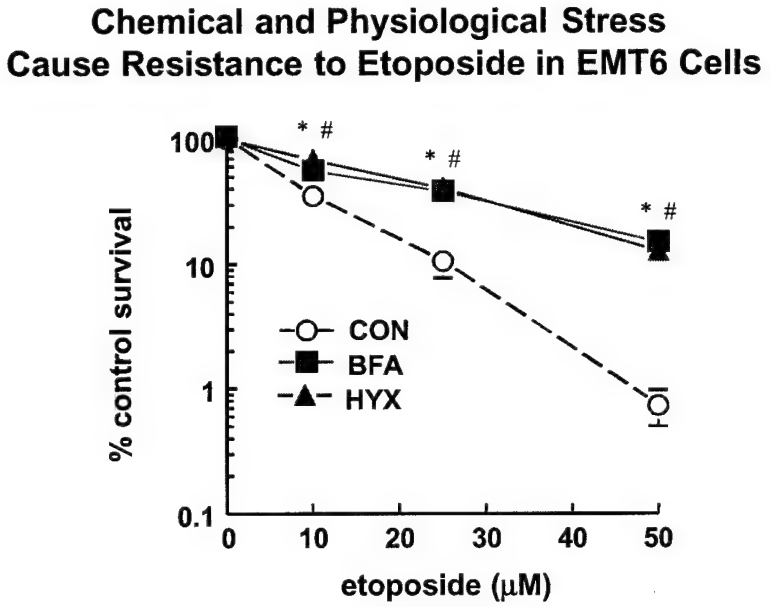


Figure 2. Expression of IκBαM prevents stress-induced resistance to etoposide. EMT6 cells transfected with an inducible IκBαM expression vector were treated with 0.1, 1.0 or 10 μM ponasterone A (PON) for 24 h to induce transgenic expression. Cells were treated in the presence of PON with 10 μg/mL brefeldin A (BFA) for 2 h followed by a BFA-free recovery for 6 h or with hypoxia (HYX) for 8 h. During the last hour of PON and stress treatment, cells were treated with 50 μM etoposide prior to analysis by colony forming assay. Results shown are the mean percent control cell survival ± SEM from 3-5 independent experiments. *, a statistically significant decrease in cell survival was observed in stress-treated IκBαM cells treated with PON compared to IκBαM cells not treated with PON ($p<0.05$, ANOVA).

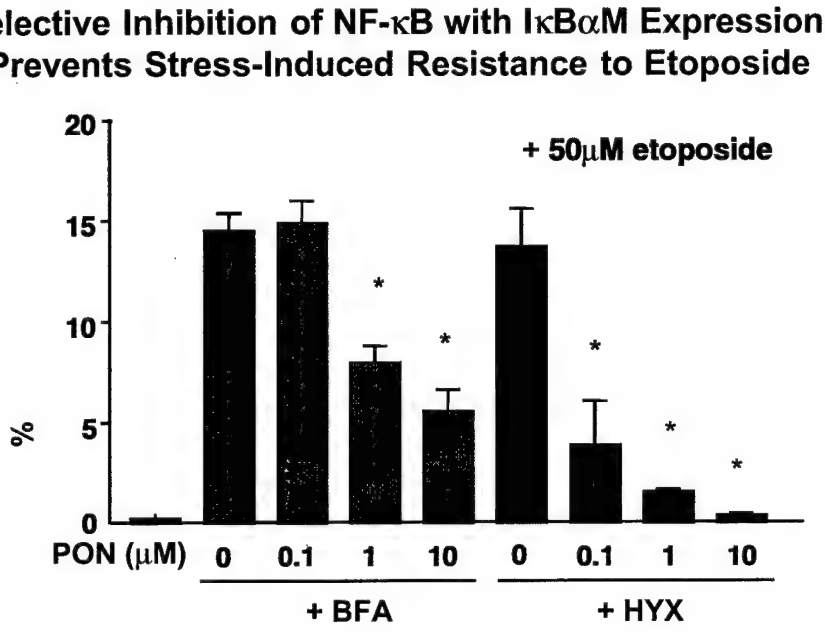


Figure 3. Expression profiles of chemical and physiologic stress. Vector-transfected EMT6 cells were treated with 10 μM ponasterone A (PON) for 24 h and either 10 μg/mL brefeldin A (BFA) for 2 h followed by a BFA-free recovery for 6 h or hypoxia (HYX) for 8 h in the presence of PON. Each expression profile was performed in duplicate and compared to another duplicate profile by a 4-way pair-wise comparison to determine the genes altered with either BFA or HYX stress. Shown are the fold-control changes in expression (2-fold and greater) with stress (BFA and HYX) of the genes (geneID) similarly up- or down-regulated during both BFA and HYX stress responses. Also shown is the Affymetrix probe set identification number (probe set) for each gene.

Genes Commonly Regulated During Brefeldin A and Hypoxia Stress

probe set	geneID	BFA	HYX	
M27500_f_at	protamine	19.3	5.0	
AF015309_s_at	nucleolar protein (MSP58)	17.9	2.7	
M95200_s_at	vascular endothelial growth factor	17.2	19.2	
L13622_s_at	s-adenosylmethionine synthetase	16.2	11.4	
M64863_s_at	cytochrome P450 17-alpha hydroxylase C17/20 lyase	15.5	11.6	
AF000581_s_at	p300/CBP/Co-Integrator protein	8.4	2.3	
L28117_s_at	NF-kappaB (p105)	7.6	2.8	
J03962_s_at	acetylcholine receptor-associated protein	7.0	5.4	
Msa.1690.0_at	I-kappa B alpha	6.6	6.2	
M12572_at	heat shock protein (hsp68)	6.1	6.2	
L06443_s_at	putative transforming growth factor-beta	5.6	5.3	
U26188_s_at	B61	4.9	3.5	
L31783_s_at	uridine kinase	4.7	4.2	
L21221_s_at	proprotein convertase 4 (PC4)	4.4	2.0	
U77040_s_at	LIM protein 3 (mSLIM3)	4.3	2.8	
AB009287_s_at	macrosialin	3.4	2.7	
AF006688_at	peroxisomal acyl-CoA reductase (muspaox)	3.4	2.6	
D37793_s_at	synaptotagminII/IP4BP	3.4	2.9	**up with**
U92794_s_at	alpha glucosidase II beta subunit	3.4	2.1	**BFA/HYX**
L04128_f_at	ribosomal protein L18 (rpL 18)	3.3	3.3	**stress**
L19737_f_at	H^+ ATP synthase subunit c	3.1	4.0	
M37761_s_at	calcyclin	2.9	2.9	
M99054_s_at	acid phosphatase type 5	2.9	4.6	
D82019_s_at	basigin	2.8	2.8	
L14677_s_at	Epoc-1	2.8	2.0	
U73445_s_at	dihydrolipoamide dehydrogenase (Dld)	2.8	2.2	
U16162_s_at	prolyl 4-hydroxylase alpha (I)-subunit	2.7	3.4	
U59807_f_at	cystatin B (Stfb)	2.7	2.3	
D44443_s_at	dexamethasone-induced product	2.6	2.0	
AF022992_at	Rigui	2.5	3.5	
D13738_s_at	putative receptor tyrosine kinase	2.5	2.0	
U67188_s_at	G protein signaling regulator RGS5	2.5	2.0	
U28656_s_at	insulin-stimulated eIF-4E binding protein PHAS-I	2.4	2.5	
M55154_s_at	transflutaminase (TGase)	2.3	2.1	
U85489_s_at	Ah receptor-interacting protein (AIP)	2.3	2.6	
D13546_s_at	DNA polymerase alpha associated subunit	2.2	2.4	
L17076_s_at	RNA-binding protein	2.2	2.0	
U19520_s_at	vesicle transport protein munc-18b	2.1	2.8	
U30482_s_at	TR2	2.1	2.1	
D49956_s_at	8-oxo-dGTPase	-2.0	-2.2	
M32309_s_at	zinc finger protein Zfx	-2.0	-2.8	
D50494_s_at	murine RCK	-2.1	-2.6	
D37790_s_at	beta-1,4-galactosyltransferase	-2.2	-2.1	
U06922_s_at	signal transducer and activator of transcription Stat3	-2.2	-2.4	
U52524_s_at	hyaluronan synthase 2 (Has2)	-2.2	-2.5	
U85614_s_at	SRG3	-2.2	-2.4	
U63933_s_at	transcription factor IID (Tbp)	-2.3	-4.1	
U19891_s_at	putative CCAAT binding factor 1 (mCBF)	-2.4	-2.2	
M31810_s_at	2',3'-cyclic-nucleotide 3'-phosphodiesterase	-2.5	-2.9	
U92437_s_at	mutated in multiple cancers protein MMAC-1	-2.5	-2.1	
U70622_s_at	lysophosphatidic acid receptor (vzg-1)	-2.7	-2.4	
D38417_s_at	arylhydrocarbon receptor	-2.9	-3.3	**down with**
M22326-2_s_at	growth factor-induced protein zif/268	-2.9	-4.8	**BFA/HYX**
U44088_s_at	TDAG51	-3.0	-4.3	**stress**
L29479_s_at	serine/threonine kinase (sak-a)	-3.1	-2.6	
M63650_s_at	M-twist	-3.2	-2.1	
U44725_s_at	mast cell growth factor Mgf	-3.3	-2.3	
U20238_s_at	GTPase-activating protein GAPIII	-3.6	-2.2	
U16322_s_at	basic transcription factor MITF-2B	-3.7	-3.2	
D78644_s_at	DNA polymerase	-3.9	-2.0	
U18869_s_at	mitogen-responsive phosphoprotein p96, p67, p93	-4.2	-2.6	
D45210_f_at	zinc finger protein	-4.9	-2.1	
U27177_s_at	p107	-4.9	-2.7	
M69293_rc_at	Id-2	-5.2	-4.6	
L02526_s_at	protein kinase MEK-1	-5.4	-7.9	
M84607_s_at	platelet-derived growth factor-alpha receptor (PDGFRa)	-5.6	-5.3	
L35307_s_at	transcription regulator c-krox	-8.7	-3.7	
M64086_s_at	spi2 proteinase inhibitor (spi2-eb1)	-9.6	-7.8	
M93422_s_at	adenylyl cyclase type VI	-10.2	-9.8	

Figure 4. Brefeldin A and hypoxia treatment alter protein levels as suggested by expression profiling. EMT6 (EMT6) or MDA-MB231 (MDA) cells were treated with 10 μg/mL brefeldin A (BFA or B) for 2 h followed by a BFA-free recovery for 6 h, hypoxia (H) for 8 h, or 10 μg/ml castanospermine (CAS). Following treatment, whole cell lysates were collected and analyzed by western blot. The resulting membranes were probed with anti-TGF-β1 (TGF-β), anti-PDGFRα (PDGFRα), anti-MEK1 (MEK1), anti-MEK2 (MEK2), anti-phospho-MEK1/2 (P-MEK1/2), and anti-actin (actin) primary antibodies. Basal expression of each protein in the absence of stress (C) is also shown. Results are one representative blot from four independent experiments. Immunoblot band intensities from EMT6 western blots were quantitated and reported as the fold-control change in band intensity with stress ± SEM relative to non-treated cells after correcting for variations in actin expression and background intensity (numbers below corresponding immunoblot data).

Western Blot Confirmation of Expression Analysis Data

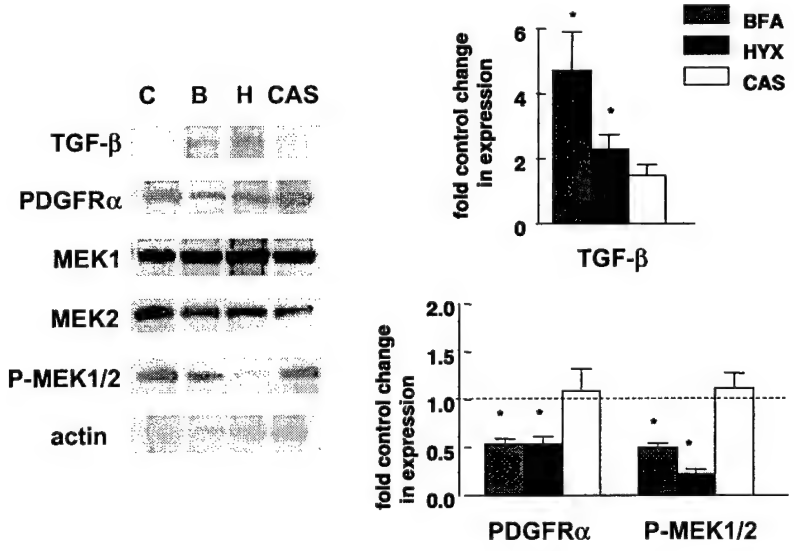


Figure 5. Treatment with TGF-β causes etoposide resistance and inhibits PDGFRα and phospho-MEK. **A.** EMT6 (EMT6) and MDA-MB231 (MDA) cells were treated with 0.1-10 ng/ml transforming growth factor-β (TGF-β) for 8 h. During the final hour of TGF-β treatment, 25-100 μM etoposide was added prior to analysis by colony forming assay. Etoposide toxicity in the absence of TGF-β treatment (CON) is also shown. Results shown are the mean percent control cell survival ± SEM from 3-5 independent experiments. *, a statistically significant increase in cell survival was observed in TGF-β-treated cells compared to CON cells ($p<0.05$, ANOVA). **B.** Whole cell lysates from EMT6 cells treated with TGF-β were analyzed by western blot using anti-TGF-β1 (TGF-β), anti-PDGFRα (PDGFRα), anti-MEK1 (MEK1), anti-MEK2 (MEK2), anti-phospho-MEK1/2 (P-MEK1/2), and anti-actin (actin) primary antibodies. Results shown are one representative blot from four independent experiments. Immunoblot band intensity was quantitated and reported as the fold-control change in band intensity with TGF-β treatment ± SEM relative to non-treated cells after correcting for variations in actin expression and background intensity (numbers below corresponding immunoblot data).

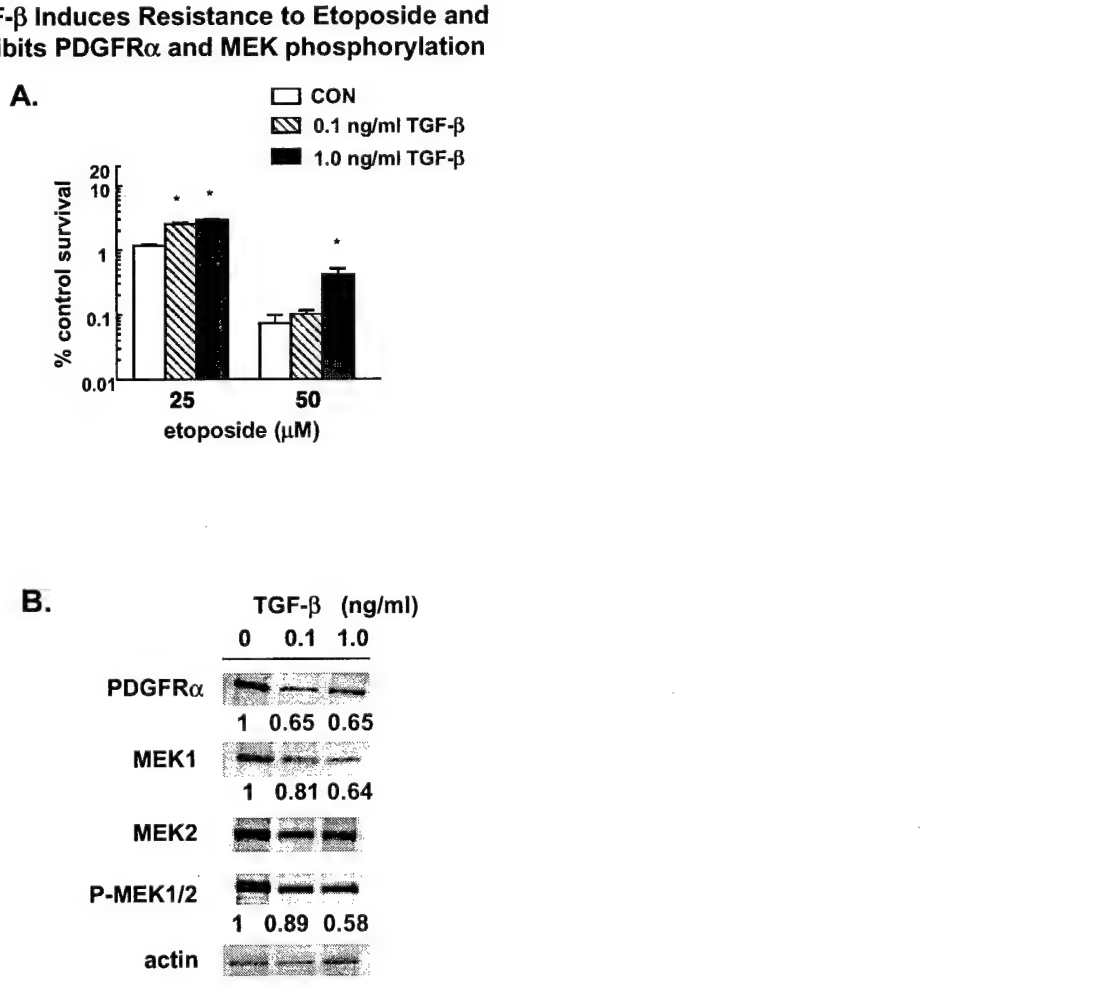


Figure 6. Treatment with anti-PDGFRα blocking antibodies causes etoposide resistance and inhibits MEK phosphorylation. **A**. EMT6 cells were treated with 1 or 10 ng/ml anti-PDGFRα blocking antibodies or pre-immune serum (pre-immune) for 8 h. During the final hour of treatment, 25 or 50 μM etoposide was added prior to analysis by colony forming assay. Results shown are the mean percent control cell survival ± SEM from 3-5 independent experiments. *, a statistically significant increase in cell survival was observed in antibody-treated cells compared to pre-immune serum-treated cells ($p<0.05$, ANOVA). **B**. Whole cell lysates from EMT6 cells treated with blocking antibodies were analyzed by western blot using anti-MEK1 (MEK1), anti-MEK2 (MEK2), anti-phospho-MEK1/2 (P-MEK1/2), and anti-actin (actin) primary antibodies. Results shown are one representative blot from four independent experiments. Immunoblot band intensity was quantitated and reported as the fold-control change in band intensity with antibody treatment ± SEM relative to preimmune serum-treated cells after correcting for variations in actin expression and background intensity (numbers below corresponding immunoblot data).

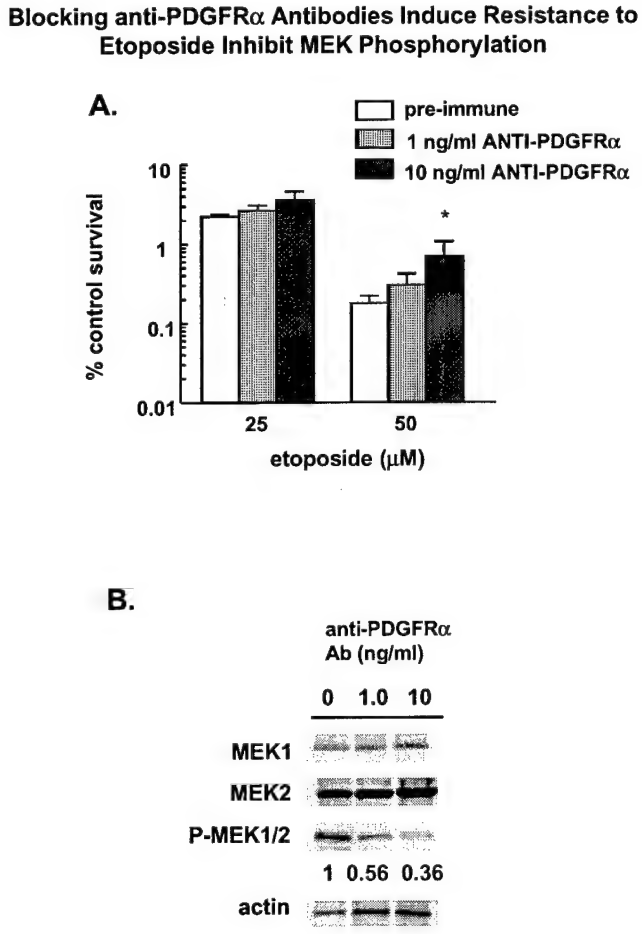


Figure 7. Treatment with U0126 but not SB203580 causes etoposide resistance and inhibits MEK phosphorylation. **A**. EMT6 (EMT6) and MDA-MB231 (MDA) cells were treated with 10 or 30 μM U0126 or 10 μM SB203580 for 8 h. During the final hour of treatment, 25-100 μM etoposide was added prior to analysis by colony forming assay. The toxicity of etoposide in the absence of stress (CON) is also shown. Results shown are the mean percent control cell survival ± SEM from 3-5 independent experiments. *, a statistically significant increase in cell survival was observed in U0126-treated cells compared to CON cells ($p<0.05$, ANOVA). **B**. Whole cell lysates from EMT6 cells treated with U0126 or SB203580 were analyzed by western blot using anti-MEK1 (MEK1), anti-MEK2 (MEK2), anti-phospho-MEK1/2 (P-MEK1/2), and anti-actin (actin) primary antibodies. Results shown are one representative blot from four independent experiments. Immunoblot band intensity was quantitated and reported as the fold-control change in band intensity with U0126 or SB203580 treatment ± SEM relative to non-treated cells after correcting for variations in actin expression and background intensity (numbers below corresponding immunoblot data).

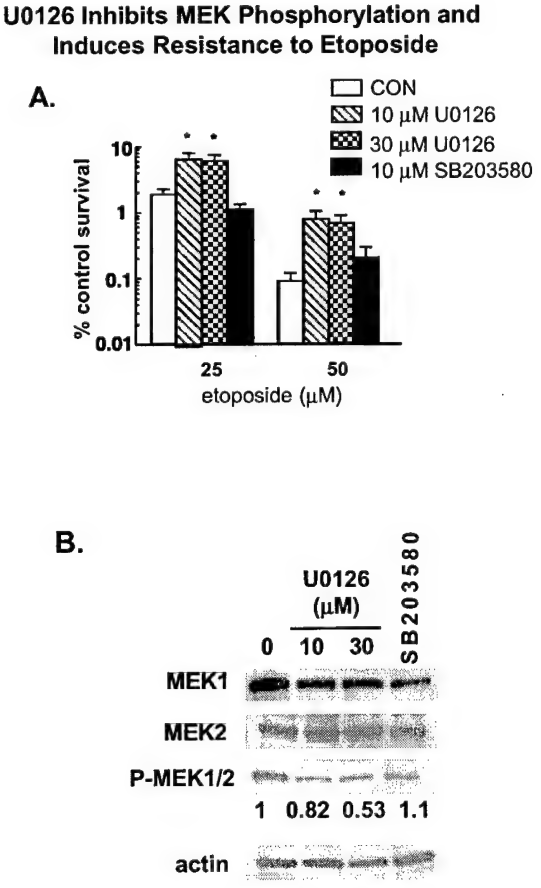
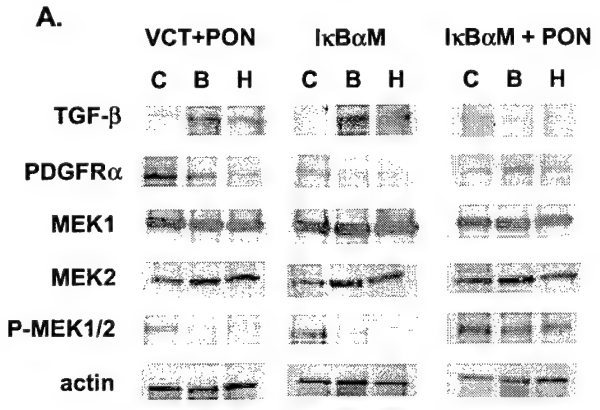
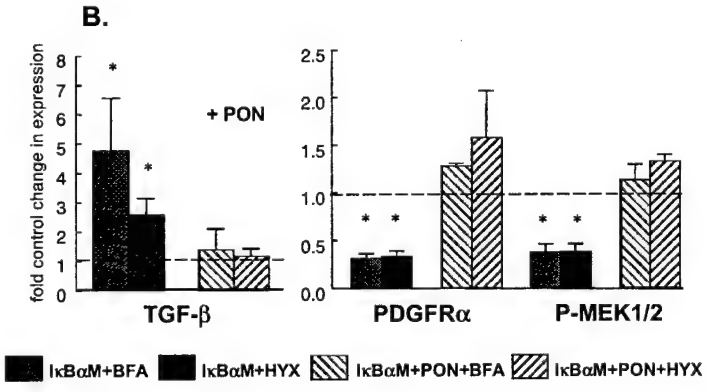


Figure 8. Stress-induced alterations in TGF-β, PDGFRα and phospho-MEK1/2 are dependent upon NF-κB activation. Vector-transfected (VCT) and IκBαM-expressing (IκBαM) EMT6 cells were treated with 10 μM ponasterone A (PON) for 24 h to induce transgenic expression. Cells were stress-treated in the presence of PON with either 10 μg/mL brefeldin A (BFA or B) for 2 h followed by a BFA-free recovery for 6 h or hypoxia (HYX or H) for 8 h. **A.** Following treatment, whole cell lysates were collected and analyzed by western blot. The resulting membranes were probed with anti-TGF-β1 (TGF-β), anti-PDGFRα (PDGFRα), anti-MEK1 (MEK1), anti-MEK2 (MEK2), anti-phospho-MEK1/2 (P-MEK1/2), and anti-actin (actin) primary antibodies. Basal expression of each protein in the absence of stress (C) is shown. Results shown are one representative blot from three independent experiments. **B.** Immunoblot band intensities were quantitated and reported as the fold-control change in band intensity with stress treatment ± SEM relative to non-treated cells after correcting for variations in actin expression and background intensity. *, a statistically significant change in expression was observed in IκBαM cells induced with PON compared to non-induced IκBαM cells ($p<0.05$, ANOVA).

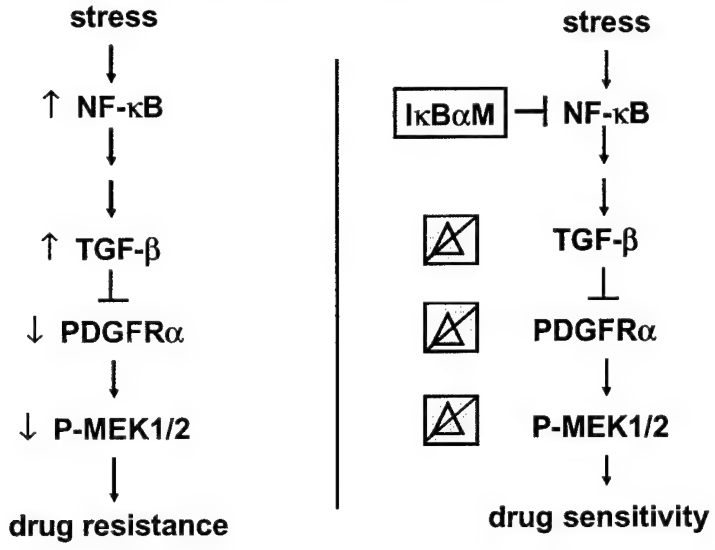
Stress-Induced Modulation of TGF-β, PDGFRα and P-MEK1/2 is Dependent Upon NF-κB Activation





Summary and Conclusions

Putative Mechanism for the Development and Prevention of Stress-Induced Drug Resistance



Expression profiling of chemical and physiological stress treated breast tumor cells reveals a signaling pathway for drug resistance throught NFkB activation, LM Brandes, DA Stephan, K. Peterson, and KA Kennedy,

Era of Hope Meeting 2002.

Abstract

EMT6 mouse mammary tumor cells treated with the physiological stress, hypoxia or with brefeldin A (BFA), an inhibitor of ER-to-Golgi protein trafficking, are resistant to the cytotoxicity of cancer chemotherapeutics that inhibit the enzyme topoisomerase II (topoII). BFA or hypoxia treatment cause activation of numerous stress pathways, including the ER-overload response (EOR) which results in activation of the pleiotropic nuclear transcription factor NF-κB. Here we show that inducible expression of a mutant IκBα protein (IκBαM) inhibits stress-induced NF-κB activation and prevents the development of BFA- and hypoxia-induced drug resistance in a manner dependent upon the extent of IκBαM expression. To further elucidate the mechanism of stress-induced resistance through NF-κB activation, we have performed gene expression analysis of BFA- and hypoxia-treated IκBαM cells using the Affymetrix GeneChip® system. Our data show several genes are similarly regulated in the reversal of both BFA- and hypoxia-induced resistance to topoII inhibitors, including genes involved in cell adhesion (e.g. α7 integrin, versican) and growth of breast tumors (e.g. FNK kinase, E4 glycoprotein, 4-1BBL, MIP-2). These data suggest that these genes represent a subset of stress-regulated genes that are likely to be involved in mediating the drug-resistant phenotype. In contrast, altered expression of apoptosis-related genes (e.g. GADD45, TRAF1, bcl-2, caspase-3) and genes regulating cell cycle progression (e.g. p53, p21, cyclin E) are selectively expressed in the reversal of either BFA- and hypoxia-induced drug resistance but not in both. Taken together, our findings support the involvement of NF-κB activation in stress-induced resistance to topoII inhibitors and imply activation of NF-κB by stress results in the modulation of multiple signaling pathways that prevent drug-dependent cytotoxicity.

INTRODUCTION

Most solid tumors are resistant to conventional cancer chemotherapies. Clinical drug resistance is caused, in part, by physiological stresses associated with the solid tumor microenvironment such as low pH, glucose deprivation, and hypoxia. Tumor drug insensitivity can be reproduced in the laboratory with chemical stress agents that mimic biological stress. We have previously shown that treatment with the chemical stress, brefeldin A (BFA), or the physiological stress, hypoxia, causes EMT6 mouse mammary tumors to develop resistance to the topoisomerase II (topoII) inhibitors etoposide and doxorubicin. Stress treatment with hypoxia or BFA is known to activate numerous stress-responsive proteins, including the nuclear transcription factor NF-κB. To determine the role of NF-κB in stress-induced drug resistance, we have introduced a mutant inhibitor of NF-κB, IκBα (IκBαM, S32/36A), into EMT6 cells to selectively block NF-κB activation. To further elucidate the mechanism of stress-induced resistance through NF-κB activation, we have performed gene expression analysis of stress-treated IκBαM cells using the Affymetrix GeneChip. Taken together, our findings support the involvement of NF-κB activation in stress-induced resistance to topoII inhibitors and imply activation of NF-κB by stress results in down-regulation of genes responsible for drug-dependent cytoxicity. These data imply that inhibition of NF-κB activation in tumors could enhance the clinical efficacy of anticancer drugs.

MATERIALS AND METHODS

Cell Culture. EMT6 mouse mammary tumor cells provided by Dr. Sara Rockwell (Yale University) were grown in a monolayer in Waymouth's MB 752/1 medium with L-glutamine (GibcoBRL, Grand Island, NY) supplemented with 15% fetal bovine serum (Sigma, St. Louis, MO), 100 units/ml streptomycin, and 25 μg/ml gentamicin sulfate (Biofluids, Rockville, MD). These cells were maintained in a humidified atmosphere of 5% CO_2/95 % air at 37°C and passaged every 3-4 days.

Reagents and Treatments. For chemical stress treatments, cells were incubated with 10 μg/ml brefeldin A (BFA) (Sigma, St. Louis, MO) for 2h followed by a 2h recovery in BFA-free media (gel shift assay) or for 2h followed by a 6h recovery in BFA-free media (colony forming assay, RNA collection) or with 60 nM OA okadaic acid (Calbiochem, La Jolla, CA) for 8h (all experiments). For physiologic stress treatment, cells were grown in glass flasks and then exposed to a humidified mixture of 95% N_2, 5% CO_2 (certified O_2<0.05ppm) at 37°C for 2h (gel shift assay) or 8h (colony forming assay). To induce gene expression, transfected cells were treated with ponasterone A (PON) (Invitrogen, San Diego, CA) for 24h. For cell survival experiments, cells were treated with 10-50 μM etoposide during the last hour of stress and PON treatment prior to colony forming assay.

Clonogenicity Assay. Following drug treatments, cells were harvested with trypsin and serially diluted in Waymouth's medium as previously described (Lin et al., 1998). For each treatment, cells were in triplicate tissue culture dishes and stained with crystal violet after a 7d incubation under maintenance conditions. For each treatment, the percent control cell survival was determined by dividing the cell survival of drug-treated cells by the cell survival of appropriate non-treated or solvent-treated cells.

Inducible IκBαM Transfection. To selectively block NF-κB activation, we selected EMT6 cells transfected with a phosphorylation site-deficient mutant of IκBα (IκBαM, S32/36A, provided by Dr. Michael Karin, Univ. of Calif., San Diego) (DiDonato et al., 1996) or a control vector lacking IκBαM (VCT) using the ecdysone-inducible expression system (Invitrogen, San Diego, CA). The IκBαM gene was ligated into the pIND plasmid and then transfected into EMT6 cells with the pVgRXR plasmid using lipofectamine reagent (Promega, Madison, WI) as previously described (Brandes et al., 2001). Following selection in hygromycin (GibcoBRL, Grand Island, NY), antibiotic-resistant colonies were expanded and screened by western blot for inducible IκBαM expression. Cell lines were maintained in Waymouth's media with hygromycin and grown in Waymouth's media without hygromycin for 24h prior to experimentation.

Electrophoretic mobility shift assay. EMSA (gel shift assay) was performed as previously described (Lin et al., 1998). Following drug treatment, cells were washed in cold PBS and lysed in 100 μl lysis buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, 1 mM DTT, 0.5% NP-40, 0.5 mM sodium orthovanadate, 1 mM PMSF) on ice for 5 min. Cell nuclei were separated by centrifugation at 5,000 rpm for 5 min at 4°C and then washed with 500 μl washing buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, 1 mM DTT, 0.5mM sodium orthovanadate, 1mM PMSF). The nuclei were then broken by three freeze-thaw cycles in an ethanol-dry ice bath. Protein concentration was determined using the micro BCA method (Pierce, Rockford, IL). To construct the probe, 3.5 pmole of oligonucleotide containing the NF-κB consensus sequence (Promega, Madison, WI) was incubated with 1 μl of [γ-^{32}P] ATP (10 mCi/ml, 6,000 Ci/mmol, Amersham-Pharmacia, Arlington Heights, IL), 5 units of T4

polynucleotide kinase (Promega, Madison, WI) and 10 μl of end-labeling buffer at 37°C for 1 h. The reaction was terminated with 90 μl 1X STE buffer (Sigma, St. Louis, MO) and then passed through a G-25 spin column (Worthington Biochem, Lakewood, NJ). Nuclear protein extracts (15-20 μg) were incubated with 3 μg poly dI·dC and 0.035 pmole of radiolabeled oligonucleotide (100,000-200,000cpm) in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 4% glycerol) at room temperature for 20 min. The binding mixtures were loaded on a non-denaturating 6% polyacrylamide gel (30:1 acrylamide:bisacrylamide, 0.5 X TBE, 2.5% glycerol) and separated in 0.5 X TBE buffer at 110 Volts for 3 h. The resulting gel was transferred to filter paper, dried under vacuum pressure, and exposed to X-ray film.

Expression analysis with the Affymetrix GeneChip. Expression profiles of stress-treated VCT and IκBαM cells were generated using the Affymetrix GeneChip system as previously described (MacDonald et al., 2001). Briefly, total RNA was isolated from stress-treated VCT and cells using TRIzol reagent (Sigma, St. Louis, MO) per product insert and converted to double-stranded cDNA using the SuperScript Choice System (Life Technologies, Rockville, MD) and a T7-$(dT)_{24}$ primer (GENSET, La Jolla, CA). Following phenol/chloroform extraction and ethanol precipitation, cDNA was converted to biotin-labeled cRNA using the ENZO BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY) and purified with the RNeasy mini kit (Qiagen, Valencia, CA). Purified cRNA was fragmented in 8 μl of 5X fragmentation buffer (200 mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc) at 94°C for 35 minutes. Fragmented cRNA (15 μg) was mixed with 5 μl of 3 nM Control Oligonucleotide B2 (Affymetrix, Santa Clara, CA), 15 μl of 20X Eukaryotic Hybridization Controls (Affymetrix, Santa Clara, CA), 3 μl of 10 mg/ml Herring sperm DNA (Fisher, Pittsburgh, PA), 3 μl of 50mg/ml acetylated bovine serum albumin (GibcoBRL, Rockville, MD), and 150 μl of 2X Hybridization Buffer [200 mM MES (Sigma, St. Louis, MO), 2M (Na^+), 40 mM EDTA, 0.02% Tween 20] and were incubated at 99°C for 5 min and then at 45°C for 5 min. Probe integrity was first assessed by hybridization to a Test2 Probe Array (Affymetrix, Santa Clara, CA) and detection with a streptavidin-labeled fluor and confocal laser scanning accoring to the manufacturer's recommendations. Verified cRNA probe was then hybidized to the Mu11K Muring Gene Array (Affymetrix, Santa Clara, CA). The Affymetrix software extracted the relative fluorescence intensities from each element on the array. Data from each array were scaled to normalize data for inter-array comparisons. The gene expression profile of each cRNA sample was analyzed twice and compared to another profiles of another cRNA sample by a 4-way pairwise comparison. Data shown are mean fold changes in expression from these 4 compairsons ± SEM.

Statistics. For clonogenicity assays, the results shown are the average percent control survival rates ± SEM from 3-5 independent experiments with three replicates per experiment. Statistically significant changes in these data were determined using one way ANOVA analysis with multiple comparisons performed using Bonferroni's test using $p<0.05$ (Motulsky, 1995).

Fig. 1. Chemical or physiologic stress induces resistance to etoposide toxicity. EMT6 cells were treated with either 10 μg/ml brefeldin A (BFA) for 2 h followed by a BFA-free recovery for 6 h, hypoxia (HYX) for 8 h, or okadaic acid (OA) for 8 h. Etoposide (10, 25 or 50 μM) was added during the last hour of stress treatment prior to analysis by colony forming assay. The toxicity of etoposide in non-stressed cells (CON) is also shown. Results shown are the mean percent control cell survival ± SEM from 3-5 independent experiments.

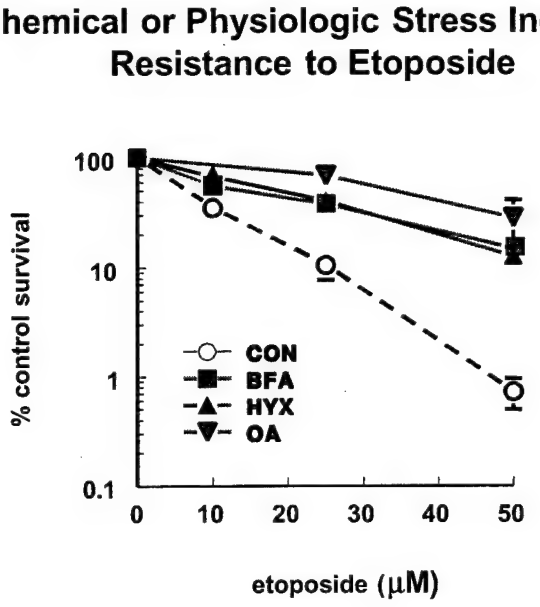


Fig. 2. IκBαM expression prevents stress-induced activation of NF-κB. VCT and IκBαM cells were treated with 10 μM ponasterone A (PON) for a total of 24 h. VCT, IκBαM, and non-transfected EMT6 cells (EMT6) were treated in the presence of PON with 10 μg/mL brefeldin A (B or BFA) for 2 h, followed by a BFA-free recovery for 2 h, 60 nM okadaic acid (OA) for 8 h, or hypoxia (HYX) for 8 h (stress treatments previously shown to cause maximal NF-κB activation). Following drug treatments, nuclear extracts from stress-treated and non-stressed (C) cells were harvested and analyzed by EMSA using a ^{32}P-labeled NF-κB oligonucleotide. Specific binding of NF-κB to the probe (NF-κB), non-specific binding (N.S.) and unbound probe (free probe) bands are indicated. The specificity of NF-κB binding was evaluated by competition assay. Nuclear extracts from non-stressed (CON), BFA-, OA-, and HYX-treated cells were incubated with oligonucleotide in the presence of a 50-fold excess of either unlabeled NF-κB or AP-1 oligonucleotide (NF-κB comp. and AP-1 comp, respectively).

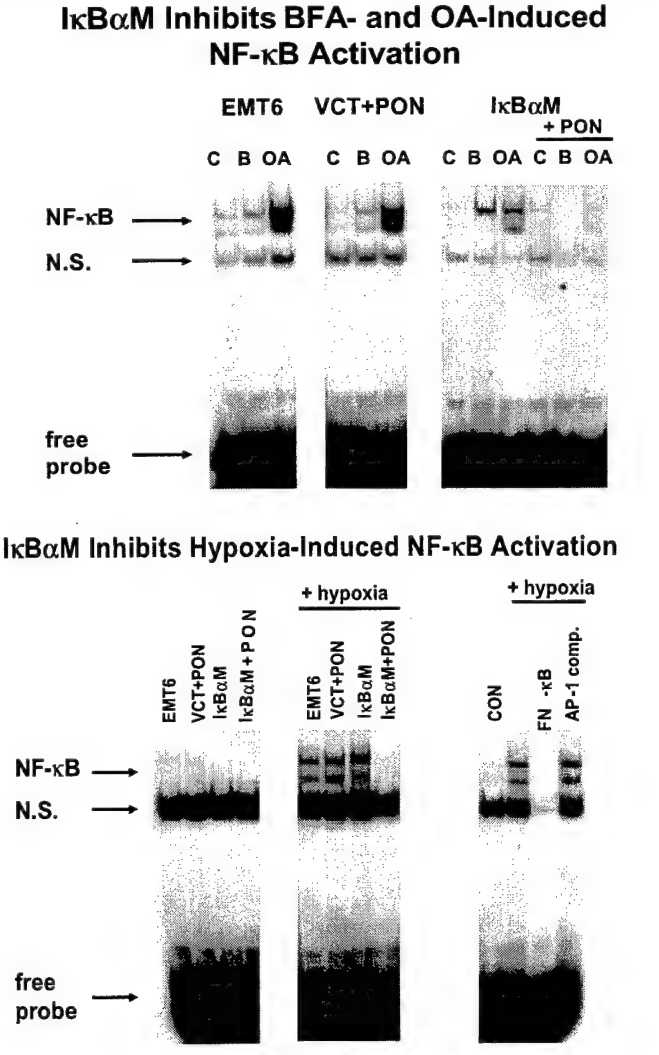
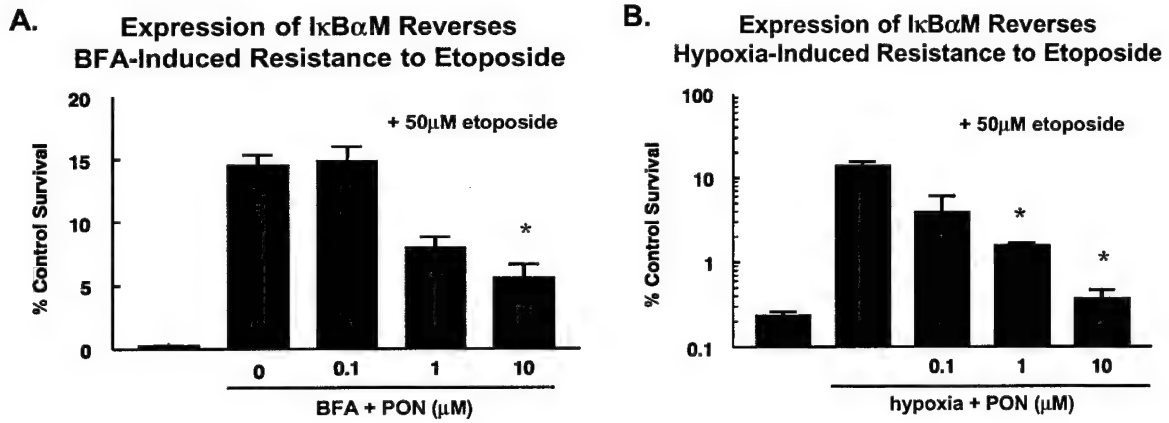
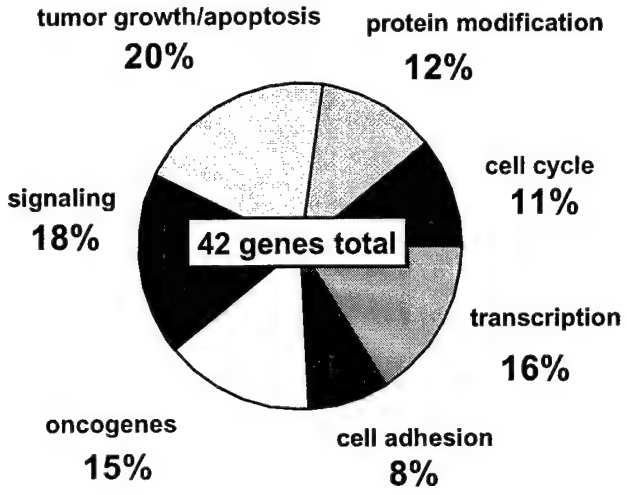


Fig. 3. IκBαM expression prevents BFA- and HYX-induced resistance to etoposide. IκBαM cells were treated with 10 μM ponasterone A (PON) for a total of 24 h. Cells were stress-treated in the presence of PON with 10 μg/ml brefeldin A (BFA) for 2 h, followed by a recovery in BFA-free media for 6 h (***A***.) or hypoxia (HYX) for 8 h (***B***.). Etoposide (10, 25 or 50 μM) was added during the final hour of PON and stress treatment prior to analysis by colony forming assay. Results shown are percent control cell survival averages of triplicate plates from at least three independent experiments; *bars*, SEM. The cell survival of IκBαM cells treated with etoposide alone is not different than that observed in VCT or EMT6 cells. There was no statistically significant change in cell survival of non-induced IκBαM cells treated with stress prior to etoposide treatment compared to VCT or EMT6 cells treated with stress prior to etoposide treatment. *, a statistically significant decrease in survival of induced IκBαM cells treated with stress was observed when compared to non-induced IκBαM treated with stress ($P<0.05$, ANOVA).



Functional Classifications of Candidate Genes Involved in BFA-Induced Drug Resistance

5-fold and greater changes in IκBαM+PON+8hBFA compared to VCT+PON+8hBFA



Candidate Genes Involved in the Reversal of HYX-Induced Resistance with IκBαM

5-fold and greater changes in IκBαM+PON+8hHYX compared to VCT+PON+8hHYX

Apoptosis-Related

caspase 3	(10.2±3.5)
TRAF2	(9.4±2.3)
GADD45	(5±3.3)
bcl-x	(-8±2.3)

Associated with tumor progression

Fli-1	(6.9±4.0)
PAI-1	(-5.6±2.1)
carboxypeptidase H	(-5.7±2.3)
cytokeratin 19	(-30.2±6.8)
nitric oxide synthase	(-50±10.3)

Cell cycle control

calcyclin	(7.1±3.2)
CDK inhibitor p15INK4b	(7.1±2.7)
TIS21	(-5±1.3)

Cell adhesion

madcam1	(5.2±2.8)

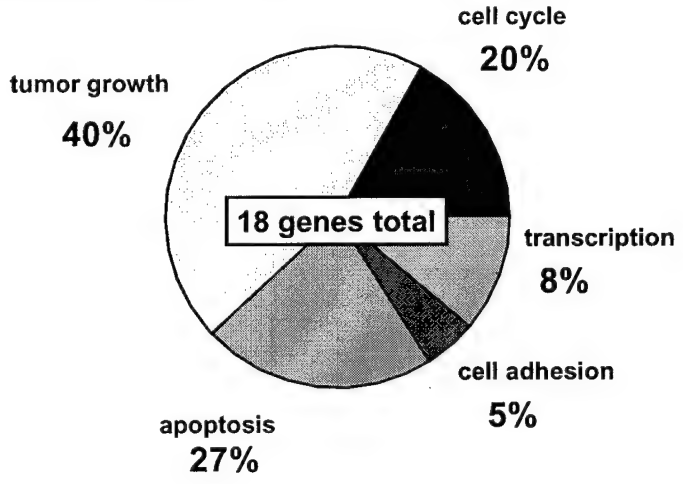
Transcription factors

PTX3	(-5.3±1.9)
junB	(5±2.9)

18 genes total

Functional Classifications of Candidate Genes Involved in HYX-Induced Drug Resistance

5-fold and greater changes in IκBαM+PON+8hHYX compared to VCT+PON+8hHYX



Candidate Genes Involved in the Reversal of BFA- and HYX-Induced Drug Resistance with IκBαM

2-fold and greater changes in IκBαM+PON+8hHYX compared to VCT+PON+8hHYX AND IκBαM+PON+8hBFA compared to VCT+PON+8hBFA

Apoptosis-Related

cytochrome c oxidase (2.8±0.7)

Associated with Tumor Progression

4-1BBL (10.1±5.3)
FNK ser/thr kinase (3.5±1.1)
HMG CoA reductase (2.9±0.8)
tumor-assoc. E4 glycoprotein (2.9±0.7)
MT-1 (-2.7±0.1)
CD13/aminopeptidase (-4.1±1.9)
versican (-5.6±2.6)
MIP-2 (-12.0±8.1)

Sensors of Free Radical Damage

MTF-1 (5.6±1.4)
MT-1 (-2.7±0.1)

Protein Assembly

Sec53p (-5.1±2.2)

Cell Adhesion

alpha7 integrin (-2.7±0.0)
integrin-assoc. prot (-3.5±1.5)
CD13/aminopeptidase (-4.1±1.9)
versican (-5.6±2.6)

Ion Channels

D3 dopamine receptor (3.0±0.9)
voltage-dep. Na+ channel (2.6±0.3)
cannabinoid receptor-2 (-2.7±0.5)

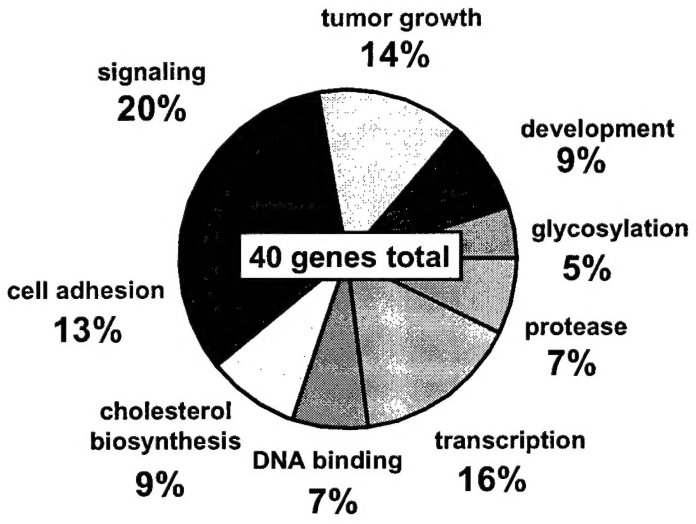
Transcription Factors

c-fos (3.9±1.3)
Sp1 (3.5±1.1)
krox20 (2.8±0.5)
sox4 (-3.2±0.3)
c-Krox (-5.5±2.7)

40 genes total

Functional Classifications of Candidate Genes Involved in Both BFA- and HYX-Induced Drug Resistance

2-fold and greater changes in IκBαM+PON+8hBFA compared to VCT+PON+8hBFA and IκBαM+PON+8hHYX compared to VCT+PON+8hHYX



Classifications of Candidate Genes Involved in the Reversal of Stress-Induced Drug Resistance with IκBαM

genes involved in reversal of BFA-induced drug resistance

apoptosis-related
cell cycle control
oncogenes

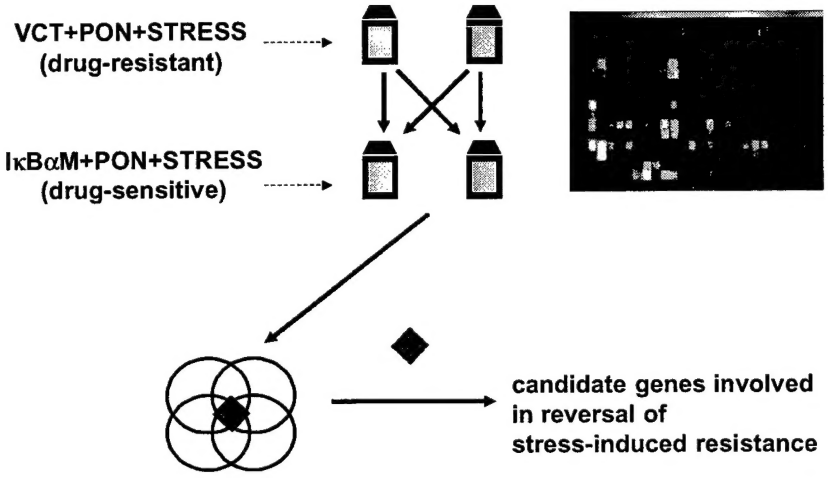
genes involved in reversal of HYX-induced drug resistance

apoptosis-related
cell cycle control
associated w/tumor progression

genes involved in reversal of both BFA- and HYX-induced drug resistance

cell adhesion
associated w/tumor progression

Expression Analysis with the Affymetrix GeneChip®



SUMMARY AND CONCLUSIONS

- IκBαM expression blocks stress-induced NF-κB activation
- Selective inhibition of NF-κB prevents the development of both BFA- and hypoxia-induced resistance to etoposide
- Genes that regulate apoptosis and cell cycle progression are implicated in the reversal of either BFA- or hypoxia-induced resistance with IκBαM expression
- Genes involved in the reversal of both BFA- and hypoxia-induced resistance with IκBαM expression are primarily associated with cell adhesion and tumor growth